METHODS OF
QUANTITATIVE MICRO-ANALYSIS

COLLECTED AND EDITED BY

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AND

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PREFACE

QUANTITATIVE micro-analysis is already a vast and rapidly expanding subject of great practical importance, and there is every indication that for many branches of chemistry it will soon become the standard procedure of the professional analyst.

Mainly in consequence of their late development, very few, if indeed any, micro-chemical techniques are as yet generally taught to the average chemistry student, and most unfortunately it has happened that many of the extremely valuable micro-analytical procedures have been developed by such extreme specialists that even the average professional analyst is not even now fully conversant with them or aware of their wide potentialities.

One of the more fortunate outcomes of "total war" in Britain has been the integration of scientific knowledge which has brought into personal contact specialists conversant with experimental techniques of different types. In consequence of their war-time experience, the Editors of this volume have been able to plan out a fairly comprehensive survey of modern micro-analytical techniques, and have been able to invite specialist contributions from practising analysts well conversant with the inherent laboratory procedures, and difficulties, of each branch of the subject. Moreover, well realising that successful *accurate* chemical analysis is even more a laboratory art than a fundamental science, they have taken care to check each section from the standpoint of the practical worker at the bench. Indeed, the original idea of preparing this volume was an outcome of friendly discussions and interchanges of laboratory techniques, and the feeling that this pooling of information should be given wider scope.

The Editors in expressing their thanks to the other contributors wish to place on record the fact that each one of them has, by spoken if not by written word, helped in the preparation of the subject-matter of more than the sections to which his name has been attached. They also wish to express their thanks to numerous other colleagues from whom specialised information or critical advice has been obtained. Particular thanks are due to Mr. M. A. Fill for his skilled work in the preparation of the illustrations and to Mr. F. P. Dunn for his care in arranging the set-out and publication of the manuscript.

R. F. MILTON

June 1948.   W. A. WATERS

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GENERAL INTRODUCTION

Editors' Foreword

BEFORE proceeding to attempt micro techniques, an analyst should have had a sound general training in chemistry and be thoroughly conversant with both the scientific principles and the standard laboratory methods of ordinary "macro" analysis. Consequently this volume has not been prepared as a manual to be followed slavishly by an assistant at a laboratory bench, but is intended as a supplement to standard treatises on the older methods of chemical analysis.

Much of the specialised technique of micro-analysis is not readily available to the average laboratory worker, and it is the object of this work to present this information in a single conveniently sized volume. A number of specialist contributors have therefore been asked to outline the types of apparatus that have been devised and the widely differing micro-manipulative procedures that are now used in the fields of inorganic, organic, and biological chemistry.

In those branches of micro-analysis in which one precise experimental technique has become well established (e.g. organic ultimate analysis) an account is given only of standardised types of apparatus of general and tested applicability. In other branches of the subject, such as volumetric or colorimetric analysis, there have been prepared concise reviews of available designs of apparatus, together with accounts of the appropriate experimental techniques. These sections of the book should be read for guidance by anyone who wishes to choose suitable laboratory equipment for specialised needs. Every micro-analyst is strongly advised to familiarise himself with a few pieces of special apparatus rather than to handle with indifferent skill a large number of alternative designs of similar equipment.

Since more comprehensive accounts of chemical principles are available in specialised treatises, the theoretical introductions to the various parts of this volume have been cut to the minimum length necessary for a rational understanding of the purposes and functions of each apparatus. Relatively more space has been, throughout, allocated to sections which indicate the chief sources of error in each type of analysis, the manipulative "snags," and the ways in which they may be overcome. It is only by acquiring this knowledge that an analytical chemist can trace his failings to their root causes and so perfect his technical ability.

Micro-analytical methods have been described in detail whenever it is essential to follow with meticulous care one rigid operational procedure (as in Parts II and VI), but in other parts of this book they have been illustrated by examples selected for the purpose of showing how various major practical difficulties can be overcome.

Quite a large proportion of these examples have been chosen from the field of biological chemistry, for there are but few comparable instances in which the professional analytical chemist will have to deal with as complex a chemical system or be forced by circumstances to work with such small quantities of material. However, many micro methods which have been elaborated into accurate techniques for the assessment of complex biological products may often be applied with equal or even greater precision in other branches of chemistry. This volume should therefore be regarded more as a compilation of micro-chemical laboratory methods than as a collection of specialised analytical procedures.

Finally, there have been included in the volume a number of tables which summarise other micro-analyses, suggest comparable procedures, and give references to the original literature. Micro-analysis is already too vast a subject for it to be practicable to include in these tables all procedures of proven value, and consequently users of this volume are advised to collect and annotate their own addenda to these tables. These summaries together with the cross-references given throughout the text should assist anyone who is searching for a micro-chemical method which is both theoretically sound and within his scope.

The Editors consider that with few exceptions they have been able to meet the requirements of all branches of analytical chemistry. The main exception, Spectrographic Analysis, is a subject so wide in scope that no contribution could be expected to cover it adequately in the available space. Furthermore, in contrast to micro-chemical applications of gravimetric, volumetric, and colorimetric techniques, quantitative spectrographic analysis is essentially dependent upon the possession of expensive specialised equipment, and is thus beyond the reach of many analysts who may be able to make regular use of the methods described in all other parts of this volume.

For similar reasons they have not, in this volume, included a section dealing with various micro-biological techniques which are now beginning to prove their value in the assays of drugs, vitamins, and other food products. It is essential that anyone who intends to use these procedures should possess, as a first requirement, a scientific knowledge of bacteriology and of its experimental techniques.

The Development of Micro-analysis

Both the principles and the techniques of analytical chemistry became standardised during the latter half of the nineteenth century. Whatever the chemical reactions concerned, gravimetric analysis involved the accurate weighing of from 0·1 to 0·5 g. of material and volumetric analysis the titration of from 10 to 50 ml. of reagent, since manipulation of quantities of the above order yielded by far the most accurate analytical results.

Work on a larger scale was cumbersome, required larger amounts of reagents, and sometimes resulted in a disproportionate increase in error.

On the other hand, accurate analysis on a smaller scale was not possible because the apparatus then available had not the required degree of precision. Other methods of analysis, such as colorimetry, were generally regarded as less accurate or of more limited application.

However, in the development of the science of chemistry there was reached, fairly early in the present century, a stage at which the adoption of a more delicate and economical analytical technique became essential before further progress in certain fields was possible. In the field of biological chemistry particularly, the advent of methods of micro-analysis was a necessary prelude to the rapid widening of our knowledge of physiological processes and the understanding of disease.

As an example of this, one may instance the need for an accurate micro-chemical method of estimation of *blood sugar*. The concentration of sugar (glucose) in the human fasting blood is of the order of 100 mg. per 100 ml. In the study of glucose metabolism it is essential to follow the blood sugar level over many hours. For this purpose small experimental animals, such as rabbits, must be used, from which the removal at frequent intervals of 50 ml. lots of blood is an impossibility. However, the development of micro-volumetric methods of sugar analysis by I. Bang* and others has enabled the estimation to be made on 0·1 ml. of blood (i.e. on 0·1 mg. of glucose) with an error of only $\pm 2\%$. Without the help of this analytical tool it would scarcely have been possible for Banting and Best to have achieved their discovery of insulin, which has so notably benefited innumerable sufferers from diabetes.

Recent progress in organic chemistry has been much furthered by the advent of micro techniques of combustion analysis, which, by enabling one to obtain an accurate elemental analysis with a few milligrams of material, have saved much time and effort in the isolation and purification of organic substances. It is generally acknowledged that without this help it would not have been possible to make any substantial progress in the structural analysis or synthesis of such important natural products as the vitamins, the hormones, and the anti-biotic drugs.

Many more examples could be quoted from the realms of industrial as well as of academic chemistry, and it is no exaggeration to say that the future of analytical chemistry will be largely furthered by the development of micro-chemical technique.

In attempting to devise micro-analytical methods, the early workers to a large extent followed the method of scaling down existing macro-chemical techniques to a diminutive level, but it was very soon realised that this simple procedure could not be followed invariably. Errors crept in from all directions, and it soon became apparent that special apparatus and special and consistent manipulative procedures were necessary before reliability could be assured. However, the modern scientific instruments

* Bang, I., "Mikromethoden zur Blutuntersuchung" (Bergman), München, 1929.

used in micro-analysis and the special techniques involved in their manipulation have now reached such a degree of precision that micro-chemical methods are quite as accurate as the older macro-chemical procedures, provided that the analyst has learned carefully and follows meticulously the correct manipulative technique.

Whilst blunders and personal errors are relatively more serious defects in micro than in macro techniques, the consequent need for a greater manipulative skill is more than compensated for by the greater overall speed which can be attained. This allows of duplicate or even triplicate analyses in the time required for a single macro-analysis.

Laboratory Organisation and General Micro-chemical Technique

After a little practice any good analyst should be able to produce satisfactory results with most micro-chemical methods, but where much of this type of work is to be carried out, some specialisation of laboratory organisation as well as of apparatus is essential.

The ideal is a laboratory specially designed for the purpose. It is helpful to have glass-topped or glazed tile benches, preferably white, with chromium-plated or rustless steel fittings. Bottle shelves should be of glass and preferably enclosed in sliding glass panels. This type of layout allows of maximum cleanliness, for it cannot be too strongly emphasised that the chief sources of error in micro-analysis are due to contamination, and that a clean, tidy bench and meticulousness on the part of the analyst are the first essentials in this field.

It is very convenient to arrange the laboratory so that particular estimations are always carried out at a given spot, and to assemble close at hand the necessary chemicals and apparatus for each operation. This apparatus when used should be cleaned and returned to the appropriate place, ready for the next estimation. Such a system may lead to some duplication of apparatus and reagents, but where micro-analysis is being carried out in routine fashion, as in biochemical, metallurgical laboratories, etc., the initial extra trouble and expense is justified.

Not only does the assembly of the necessary apparatus in one place lead to a great saving in time for the operator in carrying out each analysis, but also, by keeping the same apparatus for similar analyses, the risk of contamination by other chemicals is lessened. Again, by using the same burettes and pipettes for a particular analysis, only one preliminary standardisation is required.

Selection and Maintenance of Laboratory Apparatus
Glassware

A plentiful supply of general laboratory glassware of good quality is essential in any analytical laboratory. For micro-chemical work it is most useful to keep stocks of wide-mouthed 50 ml. Pyrex Erlenmeyer flasks and

of 100 ml. lipped Pyrex beakers, and also to have at hand a centrifuge with a supply of 15 ml. tubes. Both in gravimetric and volumetric micro-analysis much thought has been given to devising apparatus capable of yielding results of high accuracy, and the laboratory worker is strongly advised to obtain and use with due care the precision apparatus recommended in this book rather than to make shift with improvised equipment of unsuitable patterns.

After use, laboratory glassware should be rinsed without delay, cleaned with a cleaning mixture, thoroughly washed in tap water, and finally with distilled water. It may then be allowed to drain till dry on a peg-board in a dust-free room, or, more conveniently, be drained in galvanised wire baskets and dried in an electric oven. Certain precautions are, however, necessary. Under *no* circumstances should volumetric apparatus (standard flasks, burettes, pipettes) be dried in an oven or, for that matter, washed in very hot water, because glass on contraction does not return completely to its original condition; consequently heat treatment upsets the accurate calibration of glass apparatus.

After drying, the outside of glass apparatus may be wiped with a lint-free cloth, but the inside should never be touched with any wiping material. Clean apparatus should be kept in a drawer or cupboard which is dust-proof; if reassembled on a bench, then it should be covered to prevent entry of dust. Chipped pipettes and etched apparatus should be discarded.

Reagent Bottles

Reagent bottles on glass shelves should be wiped every day. A coating of ammonium chloride on bottles or shelves should not be tolerated in a micro-chemical laboratory. Fuming substances should be kept under a hood and the tops of these reagent bottles covered with small beakers.

Liquid reagent bottles should always be filled from the purest micro-analytical reagents obtainable, and solutions should in most cases be filtered carefully into the reagent bottles.

Since impurities such as dust and evaporated material tend to collect round the stoppers of reagent bottles, solutions should not be poured directly from stock bottles for immediate quantitative work. Small quantities of reagents are best withdrawn from the body of the liquid by means of clean glass pipettes or dropping tubes.

Other Laboratory Fittings

It is convenient to use modern-type retort stands, bosses, and clamps, the former of rustless steel rods in vitreous bases and the latter of a rustless alloy. Contamination due to rust or dirt falling from retort stands, girders, etc., is a very frequent source of laboratory errors.

In processes where much sampling of material is carried out, the selecting and grinding processes should be performed well away from analytical benches, particularly if the substance is of a fine, powdery nature.

Balances, colorimeters, etc., should be kept in a separate room (see p. 12). A good fume-chamber is necessary, and the prompt closing of the hood should be made an invariable routine.

Good lighting is essential in a laboratory, especially for micro-volumetric work. When artificial lighting is used, it should be from daylight lamps situated above the operator's eye-level, but arranged so that his eyes are shaded from the direct light. An overhead gas-filled or fluorescent striplight is even more effective.

Errors in Micro-analysis

In general, errors in all forms of quantitative work may be classified as (a) *determinate*, i.e. those in which the cause may be appreciated, and (b) *indeterminate*, or without apparent cause.

A systematic study of the errors in micro-analysis has been made by F. Pregl,* E. J. Conway,† and others, and the reader may find full discourses on the subject in several treatises on chemical analysis. It is nevertheless useful to emphasise the salient causes of error in quantitative micro-analysis.

Determinate errors may occur anywhere in the analytical process and may be subdivided into three groups.

Firstly, there are errors connected with the apparatus and its manipulations. Of these may be mentioned errors due to deficiencies in the balance or weights, errors due to faulty calibration, to contamination from dirty glassware, or to adsorption of materials on vessels with etched inner surfaces.

Manipulative errors may occur throughout the analytical process, the more important being:

(i) losses due to bumping when boiling a solution;

(ii) spillage during centrifuging;

(iii) loss of precipitates, which often tend to form films on the surface of the liquid or which adhere strongly to the walls of the glass vessel;

(iv) omission of temperature corrections or of calibration factors.

Impurities in reagents are frequent sources of error, and only chemicals of the highest grade should be used. Nevertheless, blank determinations should be made regularly to guard against accidental contamination.

Secondly, errors may be due to personal factors. Thus there may be errors in measurement, e.g. in the incorrect reading of a meniscus or in lack of care in the use of the balance. In some cases a constitutional factor may be present, e.g. poor colour perception or a degree of colour-blindness (which is often unrecognised).

Contamination may occur through faulty technique, such as shaking to mix a liquid in a tube closed by the thumb (see pp. 267, 334). Prejudice on

* Pregl, F., "Quantitative Organic Micro-analysis": see p. 57.
† Conway, E. J., "Micro-diffusion Methods and Volumetric Error": see p. 205.

the part of the analyst should be guarded against. Thus, when duplicate titrations are made to an indicator end-point there is a strong tendency to repeat the previous figure.

Thirdly, there may be systematic errors inherent in the particular method of analysis, and often these may be compensated for, if appreciated. Thus in precipitation reactions, the completeness of the precipitation and the solubility of the deposit in the wash liquid should both be considered. In addition, the possibility of co-precipitation must be borne in mind; while in some cases delayed precipitation may occur.

Among the more important causes of error in volumetric analyses are those due to side-reactions, auto-oxidations, and self-catalysed back-reactions. A study of the theory of the reaction often assists in minimising such errors.

Occasionally, indeterminate errors are encountered. These show as discrepancies between duplicates even when all the obvious precautions mentioned above have been observed. Only by a careful study of each stage of the analysis can they be traced to their root causes. Once detected, however, they should not be regarded as unavoidable; they should be investigated and traced to their true sources as systematic errors or as hitherto unappreciated manipulative faults.

PART I

GRAVIMETRIC APPARATUS AND GENERAL MICRO-CHEMICAL TECHNIQUES

9

10   GRAVIMETRIC APPARATUS AND TECHNIQUE

GRAVIMETRIC APPARATUS AND TECHNIQUE

MICRO-BALANCES AND THEIR USE

IN so far as it refers to the standard methods of macro-chemistry, it is generally accepted that the most accurate procedures of chemical analysis are those which make use of gravimetric techniques.

To attain an equal and consistently high standard of accuracy in gravimetric micro-analysis, great attention has to be paid to (*a*) precision in construction of apparatus and (*b*) faultless operational technique. Accuracy in the weighing of quantities of 1 to 10 mg. of material is the first essential requirement.

Whilst many special micro-balances have been constructed for particular chemical researches (e.g. the quartz micro-balances of Steele and Grant* or of Ramsay and Whytlaw Gray,† or the torsion balance of Nernst‡) none proved to be suitable for routine analysis, on account of fragility or limitations of total weighable load, until Kuhlmann, in 1906, developed an assay balance sensitive to 1 μg. (0·001 mg.) and yet sufficiently robust to carry a total load of 20 g. With this instrument Pregl was able to develop his technique of micro-combustion of organic substances, and once the practicability of micro-manipulation had been established the scope of micro-gravimetric analysis proved to be very wide indeed.

More recently, the torsion balance has been improved considerably, but its applicability is still confined to a more specialised field (see pp. 18–19 and Part III, pp. 196–200).

Errors in Weighing. Discussions of the errors involved in weighing are to be found in many standard treatises. The principal sources of error include:

(i) variations in sensitivity with change of the load;

(ii) differential temperature effects inside the balance case;

(iii) local electrostatic attractions between the insulated pans, etc.;

(iv) unequal condensation of moisture on apparatus and weights;

and, in addition, obvious technical failings, such as vibration of the balance supports and calibration errors in the replacement of one large weight by several smaller ones.

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* *Proc. Roy. Soc.*, 1909, A, **82**, 580.
† *Proc. Roy. Soc.*, 1912, A, **86**, 270.
‡ *Zeit. Elektrochem.*, 1903, **9**, 622.

The errors enumerated above are easily detected by the occurrence of a shift of the zero-point. They must be looked for continually, since they may arise, when perhaps least expected, owing to wear of the instrument or to a change in the temperature or the humidity of the room.

Error (i) is due either to the balance design or to its state of cleanliness; errors (ii) to (iv) may be due either to faulty siting of the balance or to faulty weighing technique.

The Balance-room. Unless a micro-balance is properly sited it can never give accurate results, and micro-gravimetric analysis should not be attempted if proper balance-room facilities cannot be secured. The following points should be taken into consideration in setting up a micro-balance of any type:

1. A micro-balance should be set up in a room leading off the analytical laboratory, or, alternatively, it should be partitioned off in a draught-free site.

2. The balance must be shielded from the direct rays of the sun. If possible the natural light source should be obtained from windows facing north only; failing this, the balance should be illuminated from a distant, symmetrically placed lamp. Otherwise differential temperature changes will occur.

3. The temperature of the balance-room should remain constant during any day to within $1°$ C., since balances are sensitive to temperature change.

4. The humidity of the atmosphere should be kept as constant as possible. The relative humidity should not be below 60%, otherwise trouble may be met with from electrostatic charges on apparatus. Also, deposition of moisture from condensates of steam-baths, etc., must be avoided.

5. A suitable rigid support for a micro-balance is a concrete slab mounted on supports 2 ft. 6 in. high. These should be unconnected with the walls of the balance-room and, if possible, built up from the level of the foundations, so as to avoid all outside vibrations. To damp out vibrations still further, successive strips of lead (total 1 in. thick) and a hardwood block (3 in. thick) should be placed between the concrete slab and its supports.

The Assay Micro-balance* (fig. I.1). Micro-balances are precision instruments of constant sensitivity, on which the swing of the pointer in front of an illuminated scale is observed through a short reading microscope or other magnifying device. The balance beam, which is about 70 mm. long, carries a rider scale along its full length. The latter is divided into one hundred uniform notches, numbered from 0 to 10 at every tenth notch. A 5 mg. rider is used, and the rider adjustment is provided with a lens. Each notch on the rider scale is cut so that the rider falls to the lowest point; misplacement of the rider in the notch may introduce an error of up to 3 μg.

* For a full discussion see Corwen, A. H., *Ind. Eng. Chem.* (Anal. Edn.), 1944, **16**, 258.

The sensitivity of the balance is adjusted by the maker so that, with the mean swing of the pointer at zero, a displacement of the rider by one notch produces a resultant swing of exactly ten divisions on the pointer scale. Thus 10 notches on the rider scale are equivalent to 1·0 mg.; each notch represents 0·1 mg., whilst each division on the pointer-scale represents 0·01 mg. Differences of weight of 0·001 mg. (=1 μg.) may be estimated accurately by the method of swings (see below, p. 15).

Care of the Balance and Weights. Micro-balances should be cleaned periodically to remove dust, which collects inside the case when the doors
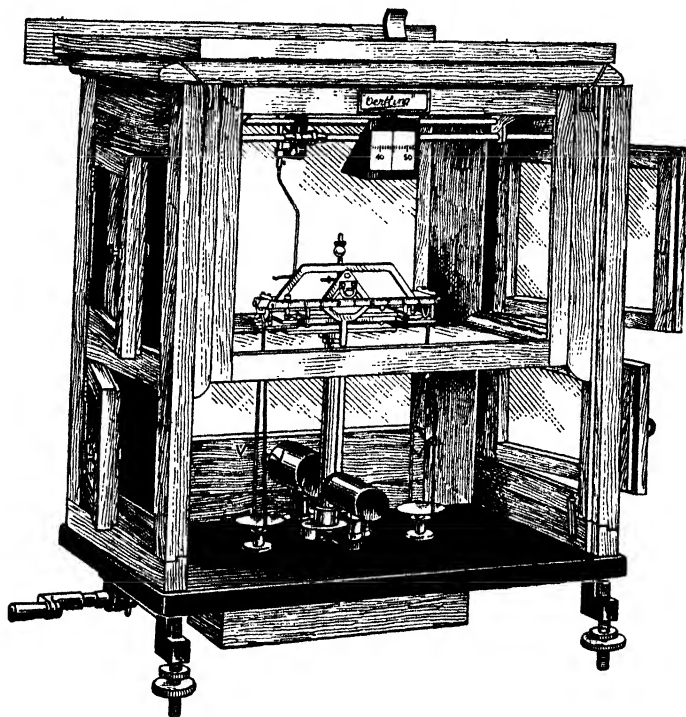
Fig. I.1 (a).   An Assay Micro-balance (Oertling Pattern).   General View.

are open. Failure to do this causes the release mechanism of the balance to stick, with the result that the pointer is jerked to one side when the arrestment is released.

In cleaning a balance the pans and their suspensions are first taken off, and then the beam is removed from the central support by holding it at the junction with the pointer.

The arresting contacts on the beam, the suspension, the central supports, and the notched rider scale are brushed with a soft camel's-hair brush;

the knife-edges are carefully cleaned with a piece of soft chamois-leather, which is used also to polish the balance pans.

The interior of the balance case should be brushed out, well rubbed with a gauze moistened with a little alcohol, and then dried with chamois-leather. After careful re-erection the balance should be tested to see that the zero-point is constant; if it is not so, then the balance should be left for a short time with the doors open to allow the evaporation of traces of moisture and the attainment of even temperature.

At frequent intervals, the small weights (best kept on a microscope slide beside the right-hand pan) should after cleaning be checked both against each other and against the rider. Any change in the sensitivity of the balance can then be corrected by means of its central adjusting screw.

Weights may be cleaned by rubbing with soft tissue paper and then brushing. They should be brushed frequently to remove adhering traces of dust.
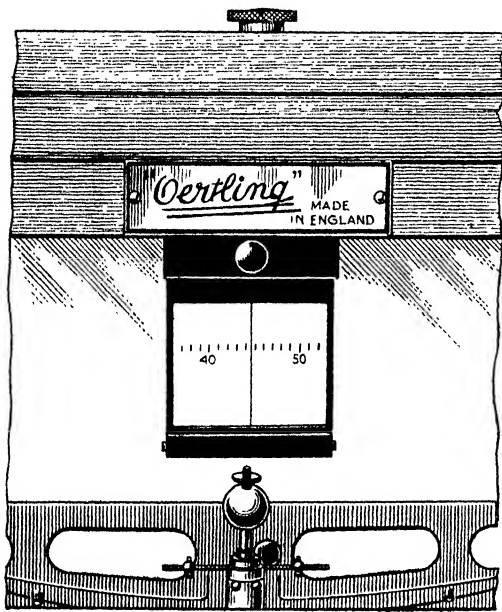
Fig. I.1 (b).   The Magnified Image of the Graticule which Functions as the Pointer.

Weighing Technique

1. All micro-chemical analyses involve weighings by difference only; i.e. the same piece of apparatus (weighing-boat, filter-tube, etc.) is weighed before and after carrying out a chemical operation.

Counterpoises (see p. 16) are therefore made so as to match both in shape and mass each piece of apparatus in regular use. Only a few small (0·1 to 0·010 g.) weights need thus be used, and consequently calibration errors are reduced to a minimum. The weights and counterpoises are kept permanently inside the balance case on clean microscope slides or on specially constructed glass stands.

2. Apparatus to be weighed, counterpoises, and weights are placed on the balance pans or on the hooks provided *through the side doors* of the balance case. The sliding front of the balance case is NOT used except for cleaning the balance, since a change in zero-point occurs on breathing directly into the balance or even from the direct heat of the body.

3. All micro-chemical apparatus must be kept inside the balance case for not less than 5 minutes before weighing, so that it attains equilibrium in temperature and surface humidity.

Glass and porcelain apparatus need previous wiping (see below, pp. 16–17) to remove traces of dust or grease and, more especially, any uneven distribution of surface moisture. Thereafter it must be handled only by means of forceps.

4. In weighing, apparatus is placed, with forceps, on the left-hand pan. The counterpoises are similarly placed on the right-hand pan, and, permitting only gentle movement of the balance beam, the rider is then adjusted until it has been placed squarely in the notch nearest to that for the correct weight.

5. The pointer is then allowed to swing freely; the first few swings are disregarded, since these will be affected by the release of the arrestment, and the terminal positions of the swings of the pointer to the left and right of the scale are then recorded for three or four complete oscillations. The swing of the balance is then arrested and the above procedure is repeated three or four times according to the

Fig. I.1 (c). The Rider and its Beam.

constancy of the balance. The exact weight is then computed as in the following example:

Rider position=2·2 mg.

Pointer Reading

	Swing	Left	Right	Difference	
	4th	8·0	5·2	2·8	
	5th	7·6	4·7	2·9	
	6th	7·3	4·4	2·9	Average=2·9 divisions
repeat	4th	5·3	2·4	2·9	to *left* of the central
	5th	5·2	2·2	3·0	zero.
	6th	4·9	2·0	2·9	
repeat	4th	6·0	3·1	2·9	
	5th	5·8	2·9	2·9	
	6th	5·5	2·6	2·9	

Hence *true weight*=(2·2 *minus* 0·029)=2·171 mg. more than that indicated by the large weights.

6. The zero-point of the unloaded balance should be checked frequently, and if necessary, corrections should be applied to weights of apparatus to compensate for any balance changes occurring between the times of initial and final weighings.

Counterpoises. Counterpoises for absorption-tubes used in combustion analysis (Part II, pp. 61, 65–68) are prepared from stoppered glass tubes of the same length and diameter (fig. I.2, *a*). They are filled with shot to just under the correct weight. Counterpoises for smaller pieces of glass or porcelain apparatus are most conveniently made in the form of small glass bottles containing shot (fig. I.2, *b*). All glass counterpoises should be distinctly numbered by etching with hydrofluoric acid.

Tare weights for platinum boats may be made from pieces of aluminium wire bent to a convenient shape (fig. I.2, *c*).

The Weighing of Glass or Porcelain Apparatus. It was proved by Pregl that it is essential to wipe all glass micro-chemical apparatus before it is weighed in order to remove the uneven film of surface moisture and microscopic traces of dust or grease. It is the experience of every micro-analyst that the exact techniques of wiping glass apparatus must be practised, and thereafter followed with meticulous care. Unless this is done unreliable results will be obtained even when all the rest of the manipulative procedure has been perfected.
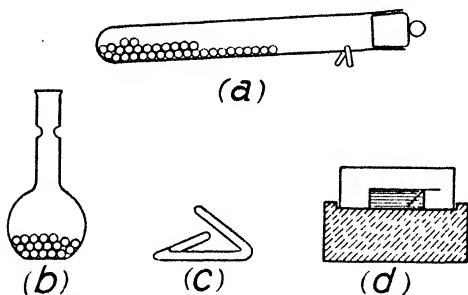
Fig. I.2 (⅓ scale).   Weighing Accessories.

In the balance-room a pair of thin chamois-leather gloves should be worn to protect apparatus from the moisture of the hand, and there should be available a supply of clean, washed (i.e. size-free) pieces of chamois-leather about 3 in. square, a selection of cotton-wool plugs mounted on short spills of bamboo, a fine camel's-hair brush, and a pocket lens.

The wiping procedure should be carried out as described below, working always in the same sequence and at the same rate.

1. *Absorption-tubes* are grasped in the centre between the thumb and forefinger of the right hand. Then (*a*) the delivery-tubes at either end are cleaned internally with a cotton-wool spill (fig. I.3, *a*); in the case of Blumer absorption-tubes (pp. 61, 63) the stoppers and the outsides of the delivery-tubes are wiped with a piece of chamois-leather. (*b*) By holding the tube at one end, the whole of the outside is wiped by careful but firm strokes in one direction only, so as not to generate electric charges. (*c*) Any excess of pressure in the tube is then released by giving the inlet stopper one complete turn. (*d*) The tube, held with metal forceps (fig. I.3, *b*) is dusted with a camel's-hair brush, examined with a lens, and if quite clean and grease-free, placed inside the balance case for 10 minutes before weighing.

2. *Filter-tubes* and other pieces of small apparatus are wiped and cooled in the same manner. Glass apparatus which has been wiped

must not be touched with the bare or gloved hand until it has been weighed.

3. *Filter-sticks and micro-beakers* should always be weighed together. (i) If a substance has to be weighed into a beaker, this and the filter-stick are wiped, after drying in an oven, and cooled for 15 minutes on a suitable cooling-block (fig. I.2, *d*). Both are then brushed; the filter-stick is placed on the hooks and the beaker on the pan of the balance, and they are weighed after standing on the balance for 5 minutes. (ii) After a reaction has been completed, the beaker only is wiped externally, without removing the filter-stick or spilling the contents. Any dust adhering to the protruding stem of the filter-stick should be removed carefully with the brush. The beaker
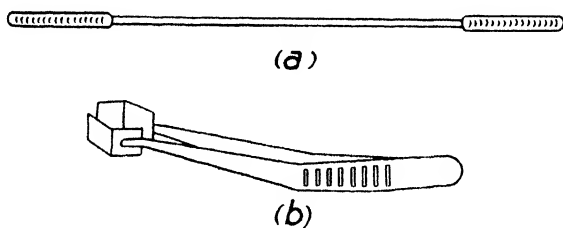
Fig. I.3. Weighing Accessories.
(*a*) Cotton-wool plug on spill for cleaning glass tubes.
(*b*) Forceps for handling glass tubes.

should then be weighed with the filter-stick inside it. Porcelain or platinum filter-sticks which have been ignited inside crucibles are not wiped before weighing, but are brushed carefully before the assembly is placed on the balance pan.

4. *Crucibles and porcelain boats* need a still longer period for cooling. They should be placed in desiccators, on copper blocks under glass covers, for 20 to 30 minutes, and then allowed to stand for 5 minutes on the balance pan before being weighed.

5. *Long-handled weighing-tubes* are often used for weighing out substances which are not hygroscopic. They are wiped after insertion of the substance, and should be left on the balance pan for 5 minutes before weighing.

THE TORSION BALANCE

A TORSION balance of the pattern indicated in fig. I.4 is a very useful acquisition in any micro-chemical laboratory, being of the most value where serial weighings of small quantities have to be made and where the required degree of precision is of the order of 1%.
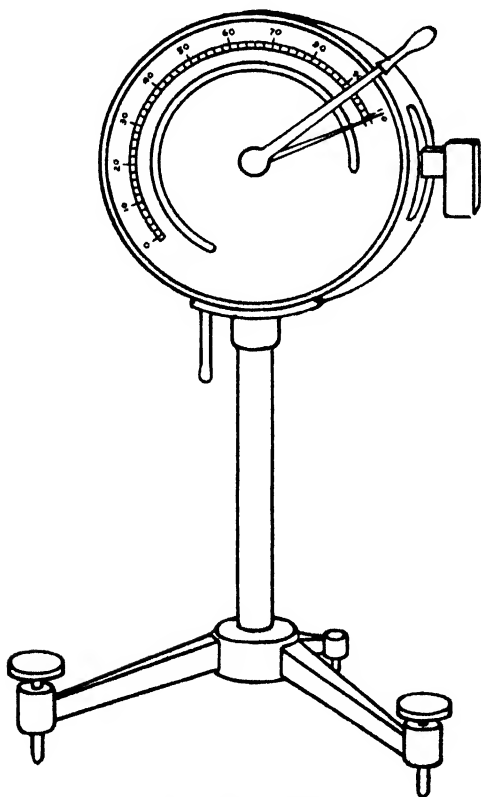
In a torsion balance, the load is applied at the end of a short lever, fixed at right-angles to a horizontal wire or fibre. The resultant twist of the wire is neutralised by an equal but opposite torque applied at the other end, and is measured on a suitable dial.

Method of Use

1. *For determination of weights of solids.* Place the solid on the pan of the torsion balance, which has previously been adjusted to zero. The weight of the substance causes the pointer to drop below the zero line; by turning the rotating arm until the pointer rises to zero again the weight of the substance may be read off on the scale (usually in milligrams).

2. *For weighing a given amount of reagent.* Adjust the instrument until the pointer is at zero. Bring the rotating arm round to the weight desired, as indicated on the scale. Add the substance to the pan until the pointer again reaches zero, when the weight in the pan will be equivalent to that indicated on the scale.

Fig. I.4. Torsion Balance.

3. *For weighing liquids* (e.g. *blood*). In place of the suspended pan, the balance is fitted with a clip which can hold a square of absorbent paper. The weight of this paper is taken in the usual way, a known volume of liquid is pipetted on to it and the paper is again weighed, the difference in weight being due to the liquid.

Applicability. The torsion balance can be used for weighing dried filter-papers containing precipitates. This is a useful procedure in laboratories where routine estimations of trace elements in raw materials are conducted. It is also valuable in cases where additions of accurately measured small quantities of a solid are necessary, e.g. in analytical procedures where addition in liquid form is inconvenient. Thus 100 mg. of a solid may be weighed on a torsion balance in a few seconds with 0·5% accuracy.

The best known application of the torsion balance is in the system of blood micro-analysis introduced by Bang (pp. 196–200). Here the absorption paper technique is used, and with one prick from the finger a number of blood samples can be made in a short space of time. Several squares of blotting paper, about 2 cm. square, are first weighed, the finger is pricked, and by means of a blood-pipette about 0·10 ml. of blood is transferred to the paper and allowed to stand for a few minutes before reweighing.

After extraction of the blood with an appropriate solvent, individual components, such as glucose, urea, uric acid, total nitrogen, fats, and cholesterol, can all be estimated. The technique lends itself most satisfactorily to micro-chemical mass-analysis.

QUANTITATIVE FILTRATION METHODS

THREE micro-chemical methods have been evolved for collecting a precipitate and determining its weight accurately. They are:

1. *Emich's "Filter-stick" Method.* In this procedure the reaction is carried out in a weighed micro-beaker of about 5 to 10 ml. capacity, the excess of liquid is sucked off through the "filter-stick" (fig. I.5, *b*), and the vessel, together with the filter and precipitate, is finally dried and weighed.

2. *Pregl's "Filter-tube" Method.* With this method, the reaction is carried out in any convenient piece of apparatus. Subsequently the whole of the material is siphoned out into a weighed filter-tube, in which the precipitate is retained on a small asbestos pad (fig. I.8, *a, b*), where it is washed, and then dried and weighed.
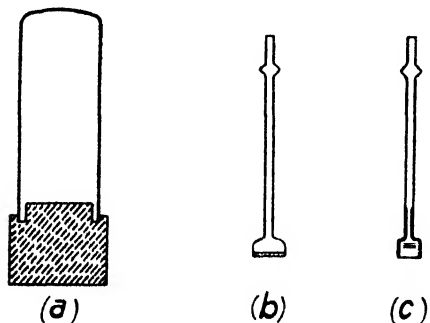
Fig. I.5 (⅓ scale). (*a*) Copper cooling block and glass cover. (*b*) Filter-stick with sintered plate. (*c*) Filter-stick with asbestos pad.

3. *Use of Micro-crucibles.* Precipitates which have to be ignited before weighing or which adhere tenaciously to the walls of reaction vessels have to be handled by the manipulative methods of macro-analysis. Small-scale Gooch crucibles (Neubauer crucibles) have, however, been developed and a suitable technique for handling quantities of precipitate of the order of 1 to 10 mg. has been perfected.

As a useful adjunct to each of these procedures, *centrifuge technique* may be used (pp. 32 to 35). Thus many compounds can be precipitated in centrifuge tubes, purified therein by repeated washing or by reprecipitation, and finally transferred to a filter-tube or micro-crucible for eventual weighing.

Other small-scale filtration devices, such as the Schwinger filter (p. 26) or the micro-dialyser (p. 37), can be used to prepare samples for analysis.

The choice of the ultimate micro-filtration method depends primarily upon the chemical form in which the precipitated material is finally weighed. Thus the filter-stick technique is the most accurate for dealing with very small quantities of material (1 to 10 mg.), but high-temperature ignition of the precipitate is not possible unless a porcelain filter-stick is used. Pregl filter-tubes or micro-crucibles should not be used for collecting less than 5 mg. of substance. The former cannot be ignited, whereas micro-crucibles can be placed inside larger platinum or quartz crucibles and heated strongly.

Thus silver halides are most conveniently collected in filter-tubes, whilst estimations of pyrophosphates or pyroarsenates must be carried out in micro-crucibles.

Filter-stick Technique. Filter-sticks (fig. 1.5, *b*) are constructed of fused quartz, Jena hard glass, porcelain, or platinum, the filtering surface being made of unglazed porcelain, sintered glass, or quartz.

The pattern of fig. 1.5, *c*, which has a removable asbestos pad, is easily made in the laboratory, and can be used reliably even for fine precipitates such as barium sulphate.

Micro-beakers should be made of hard glass. A modification incorporating the filter-stick and beaker into one vessel (fig. I.6) has been devised by Schwarz-Bergkampf.

Before use, the filter-stick is first cleaned with acid, dried, wiped, and weighed with its own micro-beaker (fig. I.7, *a*), using a counterpoise of similar shape (p. 16). The substance to be analysed is weighed directly
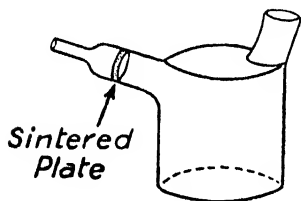
Fig. I.6 (⅔ scale).
Schwarz-Bergkampf Micro-beaker.
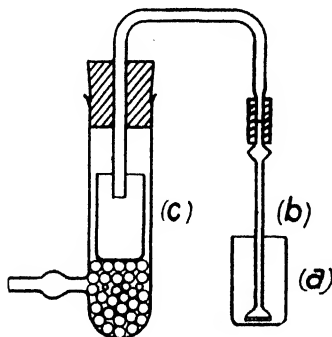
Fig. I.7.
Micro-filtration with a Filter-stick.

into the beaker and dissolved in distilled water, or alternatively a known volume of liquid is introduced from a pipette. The precipitation reaction is then carried out in the normal way, using the filter-stick, which must be inserted into the beaker only when precipitation is complete, as a stirring rod.

The precipitate is then allowed to settle, and all supernatant liquid is sucked off through the filter-stick (*b*) by attaching the latter, with rubber tubing, to a receiver of the pattern in fig. I.7, *c*. This operation must be carried out very carefully, using as little suction as possible. The residual precipitate must be washed three or four times by successive portions of wash liquid, which should be introduced from a pipette or wash-bottle down the sides of the micro-beaker and filter-stick. Finally, after sucking the residue as dry as possible, the beaker, with the filter-stick inside it, is wiped externally, placed in a small drying apparatus (the design of fig. I.16 is most convenient), and heated for the requisite time. The apparatus is finally removed, wiped carefully (see p. 16), left to cool for 10 minutes on

a copper block (fig. I.5, *a*), transferred to the micro-balance, and weighed after a further 5 minutes.

Filter-tube Technique. Pregl's design of filter-tube is shown to one-third scale in fig. I.8, *b*. It should be made of Jena hard glass, since it has been found by experience that filter-tubes cannot be made successfully of Pyrex glass, as this does not maintain a constant weight on repeated washing.

The filtering medium in the Pregl filter-tube is a pad of acid-washed Gooch asbestos, which is deposited, wet, inside the small bulb and pressed carefully into position by means of a fine glass rod. In improved designs (fig. I.8, *a*) a sintered glass plate is sealed into the tube at the base of the
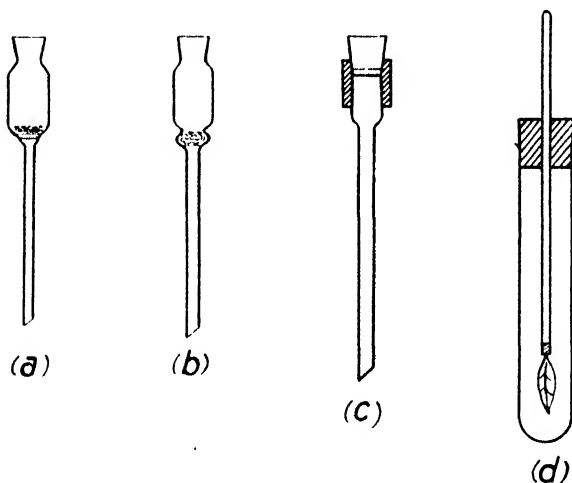
Fig. I.8.   Filter-tubes ($\frac{1}{3}$ scale).
(*a*) With sintered plate.   (*b*) Pregl's original design.
(*c*) Use of micro-crucible.   (*d*) Snipe feather.

small bulb, but the filtering surface should still be supplemented by an asbestos pad, especially if fine-grained precipitates are being handled.

The remainder of the filtration apparatus (fig. I.9) consists of a 250 ml. filter-flask fitted with a rubber stopper carrying a glass tube, about 10 cm. long and of about 1 cm. bore. The Pregl filter-tube is fitted into this glass tube by means of a stopper moistened with a trace of water. If required a small test-tube may be placed inside the filter-flask to collect the filtrate. Into the top of the filter-tube is likewise fitted a siphon-tube, made of glass capillary tubing, of about 3 mm. bore, bent twice at right-angles. For safety, this siphon should be supported by a clamp.

Before use, the filter-tube must be cleaned with hot chromic–sulphuric acid solution, washed with distilled water, and then a thin layer of Gooch asbestos should be deposited on the filter-plate from a *dilute* suspension of this material. The first operation is to wash the filter successively with

hot nitric acid, distilled water, and finally alcohol. The filter-tube is then removed, wiped with a clean cloth, and placed inside a heating block of the design of fig. I.14. A glass tube containing a plug of cotton-wool, to act as an air filter, is placed in the wide end of the Pregl filter-tube and a slow stream of air is aspirated through the apparatus as the drying proceeds. The main body of the tube should be heated to 110° C. for not less than 15 minutes.

When dry, the filter-tube is disconnected, wiped, and its weight is determined (see pp. 16–17). This should be reproducible to within 0·01 mg. after repeated washings.

In use, the filter-tube should be fitted into the siphoning system, as described above, and the precipitated material can then be carried over by applying *slight* suction at the side-arm of the filter-flask. Repeated washings with distilled water or alcohol may be needed to secure the transference of the last traces of solid. Success in this operation is most rapidly achieved by choosing as reaction vessel a small test-tube, or centrifuge tube, into the rounded bottom of which the capillary siphon-tube fits snugly. Small beakers are not convenient pieces of apparatus. The whole of the apparatus must be supported firmly, and not just held by hand.

When filtration is complete, the filter-tube is detached and then dried and weighed as already described. If necessary, the filtrate can be collected quantitatively and used for a further analysis.

The Pregl technique, which has been detailed above, is very convenient for

Fig. I.9.
Micro-filtration with a Filter-tube.

the filtration of simple inorganic compounds which form micro-crystalline precipitates. It is not suitable for collecting gelatinous substances, such as organic co-ordination compounds of metals, since these tend to adhere to the glass walls of the precipitating vessel and of the siphon-tube.

Micro-crucible Technique

(a) *General Considerations.* Convenient micro-crucibles have been designed by Neubauer: they are about 14 mm. high, 12 mm. diameter at the top, and 10 mm. diameter at the base. Crucibles made of platinum have a filtering pad of compressed platinum–iridium sponge; whilst in porcelain crucibles the base is made of unglazed material. Each crucible is provided with both a lid and a small capsule to cover the base, and should not be heated or weighed without these fitments.

For collecting precipitates in Neubauer crucibles, the apparatus used for filter-tubes (fig. I.8, c) is generally suitable. As with ordinary Gooch crucibles, micro-crucibles are held in position by a rubber sleeve, fitting over the top of the adapter tube, and filtration is conducted under *slight* suction. The rate of filtration for a good micro-crucible is about 4 ml. per minute, and this speed should not be exceeded if accurate results are to be obtained.

It was Pregl's experience* that adequate filtration conditions were attained by attaching to the side-arm of the filter-flask a rubber tube provided with a pinch-cock, and sucking gently by the mouth. This simple method is recommended strongly.

Mechanical suction should be used with great caution, since considerable practical experience is called for in acquiring adequate technical skill. If a pump is being employed, then a pressure controller should be placed between it and the filter-flask. For sensitive pressure regulation a glass tap provided with fine grooves may be used.

The Pregl siphon technique (pp. 22–23) has been advocated by Wintersteiner† for use with micro-crucibles, but considerable difficulties may be encountered when using this method, especially for fine precipitates such as barium sulphate. As the pores of the filtering surface become clogged, the filter fills rapidly, and unless great care is taken, some of the unfiltered liquid, containing precipitate, spatters over the glass cover surrounding the crucible.

Hand manipulation from evaporating dishes, as taught by Pregl, is still the safest procedure. To assist in the transference of the precipitate to the micro-crucible, a "feather" (fig. I.8, d) should be used. This consists of a small snipe feather cemented into a capillary glass tube, 12 cm. long and 2 mm. external diameter, in such a way that no liquid can enter the glass capillary. Freshly mounted feathers should be washed successively in benzene, alcohol, dilute soap solution, and finally distilled water. They should be kept in stoppered test-tubes, and must always be washed with distilled water immediately before use.

(b) *Filtration Procedure:* 1. The crucible is first cleaned in running water, using a plug of cotton-wool on the end of a match-stick. It is then placed in the sleeve of the filtration apparatus and washed several times with distilled water from a wash-bottle.

2. The crucible is then taken out, fitted with its lid and base capsule, heated with a low flame on the lid of a larger quartz or platinum crucible, and finally is ignited more strongly. When all is hot, the lid of the micro-crucible is lifted with platinum-tipped forceps, heated separately in the flame for a few seconds, and then replaced. After ignition, the crucible is removed with forceps to a copper block standing inside a desiccator and allowed to cool for 20–30 minutes. When cold, the crucible is transferred

* Pregl, "Quantitative Micro-analysis" (1924 translation).
† Wintersteiner, O., *Mikrochemie*, 1924, **2**, 14.

to another block, taken to the micro-balance, placed, with forceps, on the balance pan, and allowed a further period of 10 minutes to attain thermal equilibrium before weighing.

Platinum Neubauer crucibles require 10 minutes for cooling on the block after ignition.

3. Before filtration, the weighed crucible is mounted firmly in the rubber collar of its adapter (fig. I.8, c), which is wetted so that a good seal is obtained. The dish, or small beaker, containing the precipitate should be held in the left hand, whilst the feather should be held in the right hand. Clear supernatant liquid should first be poured into the crucible, and then suction may be applied, by mouth or from a regulated pump. When nearly all the clear liquid has been filtered, the precipitate is stirred up with the feather and transferred likewise to the crucible.

The dish is now washed down with a fine stream of wash-liquid, rubbed with the feather from the outside towards the centre, and the contents are also transferred to the micro-crucible. If the nature of the precipitate allows of it, the dish may be washed down with a fine stream of alcohol. By alternate washing with water and alcohol it is possible to take advantage of the difference of surface tension of these two liquids to detach the last traces of precipitate.

Practice is required to attain proficiency in the above procedure, and attention to the following precautions will be found to be helpful:

(i) To prevent liquid from creeping under the edge of the filtering dish or beaker, the outside of the lip should be rubbed with a finger which has been *slightly* greased.

(ii) Care must be taken that the filtering vessel never touches the micro-crucible.

(iii) The surface of the liquid in the crucible should not be touched with the point of the feather. Otherwise the surface film of precipitate will creep up the feather and much more washing will be needed.

(iv) The feather must be given a final rinse with wash-liquid and rubbed carefully in the cleaned filtering dish so as to make sure that the last traces of precipitate have been detached.

(v) Water and not alcohol should be used for the final washing.

4. When all the precipitate has been transferred to the micro-crucible, it should be washed for a few times with distilled water from a wash-bottle, and sucked as dry as possible. The crucible is then removed, wiped externally with a clean cloth, fitted with its lid and lower cover, and ignited cautiously. Procedure 2 is observed in regard to cooling and weighing. As a final precaution, it is advisable to repeat the washing of the precipitate and the ignition to check the constancy of the weight.

Precipitates which cannot be ignited should be heated in a small drying oven, such as that illustrated in fig. I.16.   In this case too the lid of the crucible should be lifted for a brief period whilst the crucible is hot.

Filtration of Crystals. Both filter-tube and filter-stick techniques can be used for collecting small quantities of recrystallised solids, obtained in the course of small-scale preparative operations or in the purification of small samples of solids prior to ultimate analysis.   Simpler filtration devices, however, can be used when the quantitative collection of all the solid is not essential.

Suction filters of normal pattern can be used in quite small-scale operations, and one simple and clean filtration device for coarse crystals is a small glass funnel with a glass bead dropped loosely into its apex.

The Schwinger filter, illustrated in fig. I.10, *a*, is very convenient for collecting quantities of a solid of the order of 5 mg. It consists essentially of a small funnel *A*, fitting flush inside rubber tubing on to a short length of capillary tubing *B*.   The interfaces of *A* and *B* are ground square and polished.   In between them is placed a circle of *hardened* filter-paper *C*, cut to correct size by means of a cork borer. Tube *B* can be fitted into any filter-flask, and the crystals, which collect in a compact plug at the narrow base of *A*, can easily be removed on to a watch-glass by means of a thin glass rod when the filter has been dissembled.
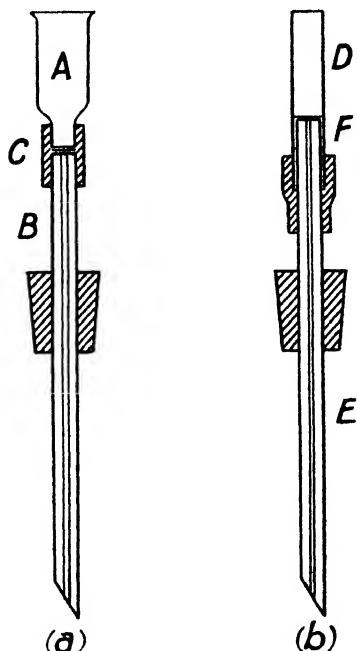
Fig. I.10 (scale ⅔).

(*a*) Schwinger Filter.   (*b*) King Filter.

Even more simple in construction is the King filter (fig. I.10, *b*), which consists of a piece of quill tubing *D*, which fits smoothly around a length of capillary tube *E*, on which rests the circle of filter-paper *F*.   This filter can be used both as a vertical filter-tube and as an immersion filter.

TECHNIQUES FOR DRYING ANALYTICAL SAMPLES AND APPARATUS

Drying of Samples before Analysis. The choice of the apparatus to be used in drying a substance before subjecting it to micro-analysis depends upon whether it is required merely to remove the last traces of a volatile solvent or whether a hygroscopic material has to be dealt with.

Many solids can be dried sufficiently well in an ordinary laboratory desiccator, but it is often advantageous and time-saving to remove the last traces of the solvent by warming the sample *in vacuo* inside an Abderhalden "drying pistol," which consists essentially of a vapour-heated tube connected with a bulb containing a drying agent such as phosphorus pentoxide, to which is fitted a tap for connection to a vacuum pump.
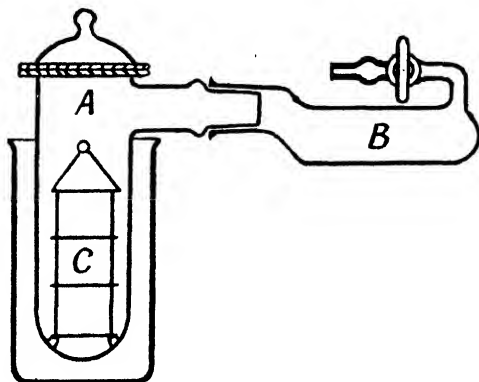
Fig. I.11. Micro-desiccator.

The all-purpose desiccator illustrated in fig. I.11 is a very convenient design of vacuum drier of this type. It consists of a Pyrex glass chamber A, rounded at the bottom, closed at the top by a ground-edged cover, and connected, by a side-arm and spring-fitted B 24 ground joint, to the desiccant bulb B. Chamber A, which can be heated in an oil-bath over a micro-burner, contains a tiered stand C, upon which can be placed small open vessels containing samples for analysis. The drying bulb B, made from wide glass tubing, is bent so that the drying agent is at a lower level than the connecting arm, and terminates at the far end with tubing bent upwards to the vacuum tap. The drying agent (calcium chloride, "anhydrone," or phosphorus pentoxide) should be well spread out along the bottom of tube B between two plugs of cotton-wool, which serve to retain any dusty particles.

For extremely hygroscopic substances a drying pistol of the pattern designed by Pregl and Roth* should be used (fig. I.12). The drying chamber A, which is surrounded by the vapour jacket B, has horizontal guide-bars C. On to these can be inserted a weighing-bottle D of square cross-section, fitted with glass vanes E, which slide over the guide-bars C. The weighing-bottle is provided with a long-handled ground-glass stopper F, so that it can be closed inside the drying tube, thus preventing any contact between the substance and moist air.

The best technique for drying hygroscopic liquids for micro-analysis is that of Alber.† The sample is dried in a small centrifuge cone, the drying agent is separated by centrifuging, and the clear liquid is then filled into a weighed capillary tube as follows. The weighed capillary tube is inserted, open end downwards, into the liquid and held in a small wire frame so that
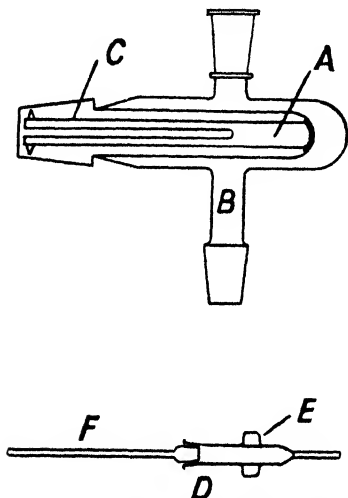
Fig. 1.12.   Pregl-Roth Drying Pistol
for Hygroscopic Substances.
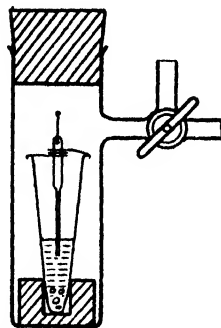
Fig. I.13.   Technique for Filling
Capillary Tubes with Dry
Liquids.

its tip is clear of the drying agent. The centrifuge tube and capillary are then placed inside a side-armed test-tube, which is then stoppered and evacuated through a drying tube provided with a glass cock or screw-clipped rubber joint (fig. I.13). If permissible, the test-tube is warmed slightly with the flame of a micro-burner. After one hour, dry air is allowed to enter the tube slowly. This forces liquid up into the capillary, which is then removed, rapidly wiped clean, and sealed at the tip by warming in a flame.

Drying of Micro-gravimetric Apparatus. Small, compact pieces of apparatus, such as Pregl filter-tubes, can best be dried by heating inside a *Pregl drying*

* Pregl-Roth, "Quantitative Micro-analysis," p. 55.
† Alber, H. K., *Mikrochem.*, 1938, **25**, 167.

block (fig. I.14). This consists of a pair of plane-faced copper or aluminium blocks, about 2·5 cm. thick and 8 cm. across, mounted on a rigid base over a micro-burner. The upper block can be removed by means of a heat-insulated handle, and the lower block is drilled to take a thermometer, mounted horizontally.

The blocks are drilled together so that each contains two parallel semi-circular channels, one of about 1 cm. diameter and the other of 1·5 cm. diameter. Filter-tubes can be dried directly inside these channels; small apparatus, such as weighing boats, are dried inside tubular *micro-desiccators* (fig. I.15), which fit into the drying block.

Micro-desiccators are easily made from glass tubing, of about 25 cm. length and 1 cm. diameter, constricted in the centre to a fine capillary. One half of the tube serves to hold platinum boats or other small apparatus needing drying. This section of the tube is closed by a rubber stopper which carries a drying-tube filled with

Fig. 1.14. Pregl Drying Block.

anhydrone and has, at its far end, a short piece of capillary tubing. The other half of the desiccator tube is filled, between cotton-wool plugs, with anhydrone, and this section too is stoppered with a bung through which passes a short piece of capillary tubing leading to a cotton-wool air filter.

When in use, the micro-desiccator is held in a Pregl heating block by means of two corks, which are flattened at one side so that the desiccator tube has no tendency to turn and spill out contents when placed on the laboratory bench. By sucking a gentle current of air, through the air filter and anhydrone drier, over the surface of a substance which is being heated,
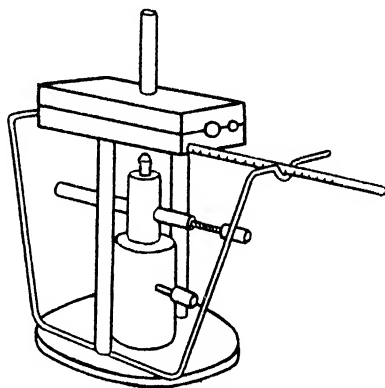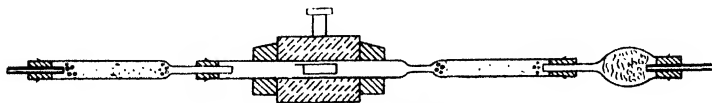
Fig. I.15 (⅓ scale). Pregl's Micro-desiccator.

all traces of moisture or solvent can be removed very rapidly. Alternatively, of course, the tube can be evacuated.

It is often desirable to use this apparatus for drying to constant weight samples for analysis. The standard procedure to be adopted in this case is to heat the substance in an air stream for half an hour and then to allow the drying block and micro-desiccator to cool for another half-hour, maintaining throughout the current of dry air over the specimen. Substances which

cannot be dried to constant weight in this way must be handled inside stoppered weighing-bottles (see p. 67).

For drying larger pieces of apparatus, such as micro-beakers and filtersticks (pp. 21–22) or micro-crucibles (p. 23), the micro-oven (fig. I.16) may be used. Alternatively, a larger micro-desiccator may be constructed from 2–3 cm. bore glass tubing. The design published by Benedetti-Pichler* is quite a useful one for this purpose.

The micro-oven (fig. I.16) is bored to a depth of 8 cm. from a cylindrical
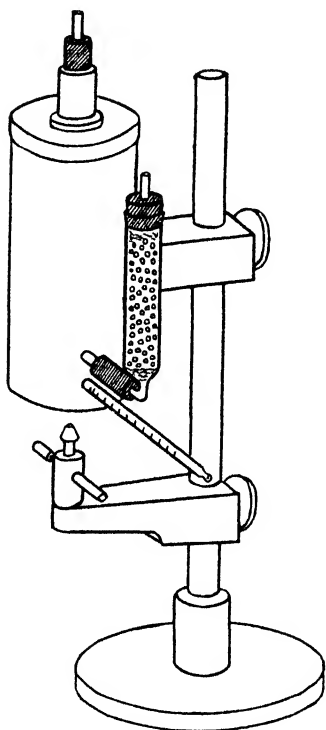
Fig. I.16.   Micro-oven.
(Designed by G. I.)
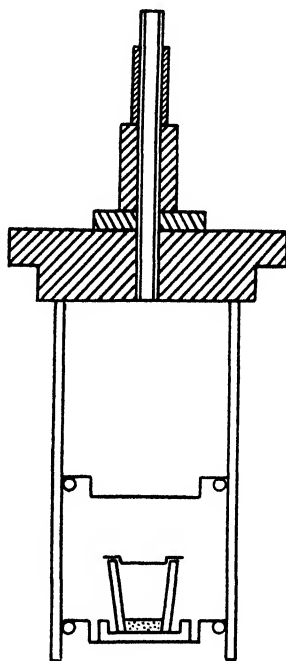
Fig. I.17 (¾ scale).   Oven Lid for Drying Crucibles.   (Designed by G. I.)

brass block, 5 cm. in diameter and 10 cm. long, which is mounted vertically on a supporting stand over a micro-burner. The main chamber has an outlet for connection to a drying tube and dust filter, whilst the solid base is bored for a thermometer. The turned metal lid (fig. I.17) carries the necessary supports for drying micro-crucibles, and also the suction lead.

Micro-crucibles may be placed in a tray and dried rapidly in a current of filtered dry air (fig. I.16).

* Benedetti-Pichler, *Mikrochem.*, 1930 (Pregl Festschrift), **6**.

The lid shown in fig. I.18 should be used for micro-beakers and filter-sticks. The beaker is held in the adjustable clip *A*, whilst the filter-stick is pushed up the suction tube *B*, until its glass flange, which is about 6 cm.
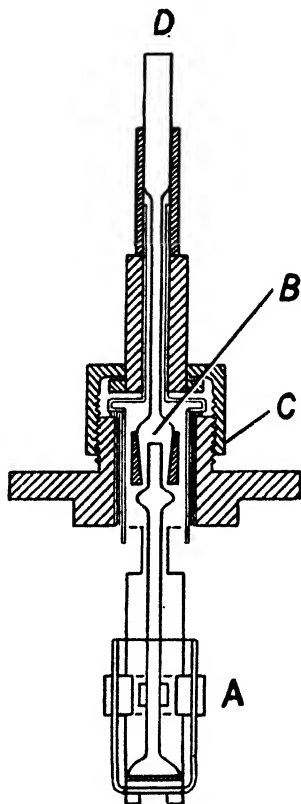
Fig. I.18 (¾ scale). Oven Lid for Drying Filter-tubes and Beaker.
(Designed by G. I.)

from the sintered plate, becomes flush with the rubber collar *C*. When suction is applied at *D* the filter-stick is held firm, though it drops free when the micro-beaker is lowered from its clip.

THE CENTRIFUGE

Principles. The centrifuge is an invaluable piece of apparatus to the micro-chemist. It is, in fact, an elegant device for the rapid separation of a solid, which enables one to deal easily with minute amounts of precipitates which in the ordinary course of events might be lost on the walls of the precipitating vessel. In biochemical analytical laboratories a centrifuge is used more frequently than any other single piece of apparatus; its extended use in other fields is strongly recommended.

The function of a centrifuge is to separate one substance from another (usually a suspended solid from a liquid) by means of centrifugal force. The mixture to be separated is placed in a centrifuge tube (fig. I.19, *a*)

(*a*)   (*b*)   (*c*)   (*d*)
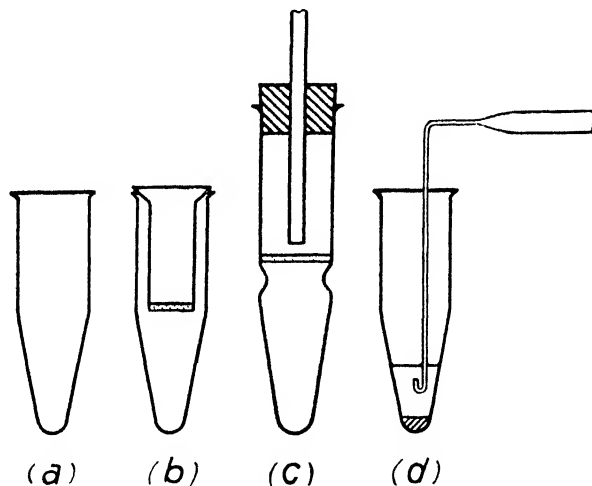
Fig. I.19.

(*a*) Centrifuge tube. (*b* and *c*) Tube for collecting a liquid by centrifuging. (*d*) Removal of a liquid from a centrifuge tube containing a precipitate.

which is fitted into a centrifuge bucket. Two, four, or eight such buckets are placed symmetrically round a spindle and made to revolve at high speed (up to 5,000 revolutions per minute), so that the buckets fly out into a horizontal plane. The centrifugal force on the suspended particles causes them to be thrown to the tapered bottoms of the centrifuge tubes and to be packed tightly together.

After centrifuging for a sufficiently long period at a suitable speed, the supernatant liquid can usually be decanted from the centrifuge tube without disturbing the precipitate at the bottom of the tube. Precipitates separated in this manner can easily be washed free from the precipitating solution by resuspending in the wash solution and recentrifuging.

It is obvious that this method of washing precipitates by resuspension is more efficient than is the simple passage of fluid over a packed precipitate, as occurs on Gooch crucibles or on filter-papers. Moreover, in centrifuging the amount of wash-liquid may be kept down to a minimum, and consequently loss of precipitate by re-solution is likewise minimised.

When it is desired to collect quantitatively a filtered liquid, a device such as that shown in fig. I.19, *b* may be used. This consists of a double tube, the inner portion of which is made of a porous material, e.g. sintered glass, pipeclay, alundum, etc., and contains the suspended liquid. On centrifuging, the liquid is thrown quantitatively through the pores of the container into the glass tube and the precipitate is retained in the filter. Alternatively the arrangement in fig. I.19, *c* may be used.

Design of Centrifuges. There are several sizes of centrifuges, but for normal micro-chemical work the most convenient is one which holds eight buckets carrying tubes of capacity from 10–15 ml. Such tubes are capable of dealing with precipitates of from 0·1–5 mg. in total weight.

There are many different models of centrifuges on the market and the reader is referred to manufacturers' catalogues for illustrations of the various types which are available. He is recommended to choose a stoutly-constructed model, with a strong spindle housed in a heavy metallic casing, fitted with a strong lid, and designed so that spilled liquid cannot penetrate into the driving motor. For safety reasons, centrifuges should never be run without the lid tightly closed.

There is always some vibration when a centrifuge is running, and consequently the instrument should be mounted on resilient rubber buffers or else slung by chains from a cradle.

Centrifuging Technique. When using a centrifuge for quantitative micro-analysis, the following points of manipulative detail should be observed:

1. A centrifuge *must always* be perfectly balanced when in use; i.e. the weights in symmetrically opposite buckets should be equal to the nearest 0·1 g. For convenience, a rough counterpoise balance should be kept proximal to the centrifuge, and to equalise the weights in opposite buckets one should proceed by adding carefully to the lighter tube some more of the liquid which is being centrifuged or else pure solvent.

2. Centrifuge tubes should not be filled to more than 1 in. from the tops of the tubes. Vortexing of the liquid occurs at high speeds and results in spillage of liquid into the buckets unless this precaution is taken.

3. Centrifuges are always fitted with variable resistances. They should be started off at low speeds and gradually worked up to the maximum required. The precaution minimises both spillage and breakage. The speed at which the centrifuge should be run depends to a large extent on the type of solvent and the degree of dispersal of the precipitate. A semi-colloidal substance in a solvent such as a protein solution requires rapid

and prolonged centrifuging; but it is rarely necessary to exceed a speed of 3,000 revolutions per minute for longer than about 10 minutes when dealing with powdery precipitates.

Centrifuging at very high speeds is usually undesirable, since it tends to pack the precipitate too closely and so make washing more difficult.   Moreover, unless the centrifuge tubes have been made from resistance glass of specially selected evenness, they are more liable to fracture when subjected to high speeds.

4. Centrifuges should be slowed down gradually at the end of the appropriate period.   They should be fitted with a free-wheel and should be allowed to rotate until they stop spontaneously, since violent changes of speed of rotation tend to disturb the precipitate.

5. To separate the liquid from the solid it is usually sufficient to decant the supernatant fluid from the precipitate by inverting the tube gradually and allowing the liquid to flow out at a moderate speed, keeping the angle of inversion consistent with the amount of liquid left in the tube.

The tube should then be inverted over a filter-paper and the residual liquid allowed to drain from the walls of the tube.   The drop of liquid which finally hangs to the lip of the tube may be removed by just touching it with a piece of filter-paper.

6. Precipitates separated by centrifuging must be purified by washing. To do this, first break up the clotted mass by holding the centrifuge tube loosely in one hand and gently tapping the bottom of the tube with the fingers of the other hand so as to splash the precipitate up the sides of the tube.   Then add the wash-liquid from a pipette in such a way that the sides of the tube are washed thoroughly.   (By using a pipette rather than a wash-bottle for this operation, equal volumes of wash-liquid may be added to each tube, and this facilitates the subsequent equalisation of weights in the tubes.)

Again flick the bottom of the tube with the fingers to ensure intimate contact between the precipitate and the wash-liquid, and finally recentrifuge.

The washing and recentrifuging should be repeated twice, making three washes in all, before it can be considered that any precipitate is completely free from the excess of the original precipitating agent.

Occasionally, certain anomalies of behaviour in the centrifuging of precipitates are encountered.   Thus with a precipitate of low density, the particles do not pack well, and then decantation by inversion would result in certain losses.   One may then resort to removal of the liquid by means of a suction pipette drawn out to a capillary and with a slightly inverted end (fig. I.19, d).   By this means the supernatant fluid may easily be removed from the tube without disturbing the precipitate beneath.

Occasionally some of the precipitate tends to form a film at the surface of the liquid and will not centrifuge down to the bottom.   This tendency

may usually be overcome by the addition of a few drops of alcohol, which will reduce the surface tension of the liquid. If this is not practicable for various reasons, then the liquid must be removed by means of a capillary pipette, taking care that the tip of the pipette is always below the meniscus of the liquid. When this technique is followed, the precipitate should be washed at least five times.

The Scope of the Centrifuge in Quantitative Micro-analysis. Centrifuge technique is helpful whenever small volumes of liquids have to be dealt with. It is invaluable for handling viscous solutions or emulsions, and also for collecting slightly soluble precipitates which would dissolve in large volumes of solvent of wash-liquid. Typical operations in which centrifuge technique has proved to be advantageous are summarised below:

1. Purification of small quantities of material, e.g. micro-crystallisation.

2. Selective adsorption of materials from small quantities of solution.

3. Separation and purification of precipitates from complex mixtures prior to dissolving up for volumetric or colorimetric analysis. This is particularly useful in biochemical analysis whenever colloidal solutions have to be dealt with. For instance, in the estimation of calcium in blood serum (see p. 167) calcium oxalate is precipitated in a centrifuge tube, purified, and finally redissolved and estimated volumetrically with potassium permanganate.

4. Collection of slightly soluble precipitates in weighed centrifuge tubes.

5. Analysis of suspensions or emulsions, e.g. estimation of cream in milk or albumen in urine by measurement of the volume of the material which is separated.*

* This type of estimation assumes that the particle size is always constant; if conditions of precipitation can be standardised, then quite accurate results are obtainable. Graduated centrifuge tubes are used for analyses of this type.

SEPARATION BY DIALYSIS
(ULTRA-FILTRATION)

Scope in Micro-analysis. In effecting the micro-chemical estimation of the inorganic constituents of biological fluids and plant extracts, many difficulties arise in consequence of the presence of colloidal matter, which interferes with quantitative precipitation reactions, adsorbs indicators used in volumetric analysis, and in many ways interferes with colorimetric processes (compare pp. 256–267).

Whilst destruction of organic matter by wet oxidation methods (pp. 100–102) or ashing is often resorted to, these procedures may often involve complications. Thus, wet oxidation may necessitate the introduction of large amounts of inorganic salts or may involve losses in the form of particulate fumes (e.g. during the evaporation of excess of sulphuric or perchloric acids) or fine dust (e.g. in dry ashing). These errors are particularly serious in estimation of the alkali metals. Appreciable amounts of these salts may be extracted from glass or porcelain apparatus in the course of high-temperature reactions.

Consequently the separation of simple inorganic ions from complex colloidal molecules by dialysis through a semi-permeable membrane is often a particularly convenient, and quantitative, purification technique. It must, however, be applied with due consideration of the nature of the material which is to be examined. Whilst dialysis will effect the removal of simple salts such as sodium chloride from organic matter, and can, in acid solution, effect the quantitative removal of sodium and potassium ions from proteins, etc., it does not break down many large colloidal molecules which contain metallic elements as part of their internal structure. Thus, by dialysis, iron is not removed from haemoglobin or magnesium from chlorophyll; whilst, again, the complex phosphoric acids of nucleoproteins, etc., may retain elements such as calcium. Metallic elements in such form would be set free only by the complete destruction of the organic matter. Again, the dialysis of earth, or clay, can only effect the partial removal of anions such as sulphate, fluoride, and phosphate.

Dialysis even under pressure or suction, however, may be extremely slow, even on the micro-chemical scale. Electro-dialysis, in which the transport of ions by an electric current passing through a solution is used to promote their diffusion through the semi-permeable membrane, is much more rapid and also much more accurate. Moreover, the completion of the separation process can easily be seen.

Experimental Methods. The method due to Nicholas,[*] using apparatus illustrated in fig. I.20, exemplifies the *pressure technique* of ultra-filtration. The apparatus consists of a cone containing a flat metal gauze on which rests a collodion or parchment membrane. This membrane is held in position by a screw ring, and the liquid to be dialysed is placed in the upper chamber, which is fitted with a Schraeder-type valve. Air is introduced by means of a foot-pump into the chamber until a pressure of about 20–30 lb. per sq. in. is recorded. This is usually sufficient to effect fairly rapid ultra-filtration. A quantity of water is introduced after a time period, to ensure that the dialysis is complete and that the dialysate is washed from the apparatus.

The method has been applied successfully to the estimation of so-called "bound calcium" in blood serum.
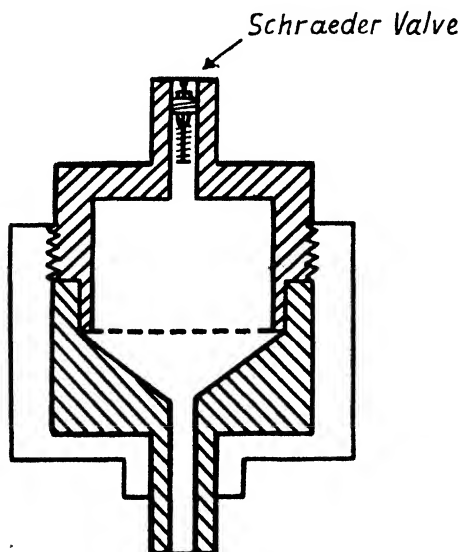
Fig. I.20. Pressure Dialyser.
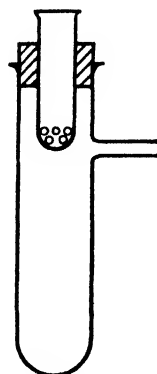
Fig. I.21.
Ultra-filtration
Apparatus.

The *suction technique* has also been used for the estimation of bound calcium. The apparatus consists of a small Soxhlet thimble which has been impregnated with a collodion solution. The thimble is held in a tight-fitting, perforated glass cylinder which is fitted to a filtration flask (fig. I.21). Application of suction to the flask allows ultra-filtration to proceed.

Where rather larger quantities of liquid are available and dilution of the dialysate permissible, the *circulating dialyser* due to Taylor *et al.*[†] may be used.

The apparatus (fig. I.22) consists of a globular funnel with a slight constriction and orifice at *A*. This is sealed to a glass rod *B*, which connects

[*] Nicholas, H., *J. Biol. Chem.*, 1932, **97**, 459.
[†] Taylor, A., Parpart, A., and Ballentine, R., *Ind. Eng. Chem.* (Anal. Edn.), 1939, **11**, 659.

to a second tube C with a similar orifice at D. The tube is then bent at right-angles and has an outlet E for removing the fluid. The tube is again bent at right-angles and continues up so as to re-enter the funnel. A device is inserted at F so as to allow a continuous stream of nitrogen, or other gas, to ensure circulation of the liquid. A semi-permeable membrane G, cylindrical in shape, is placed in position between A and C to enclose the liquid which issues from A and is returned to D. The membrane is fitted with a water-jacket with circulating water, so that the dialysate may be
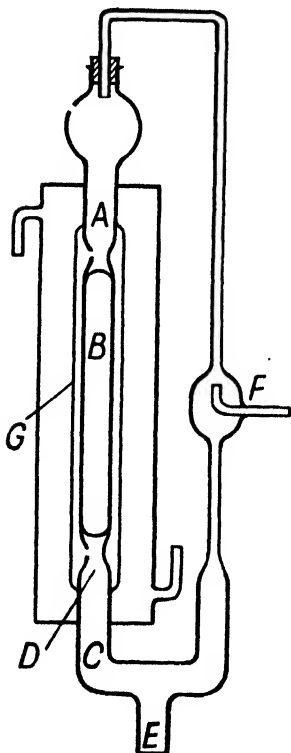
Fig. I.22.   Circulating Dialyser.
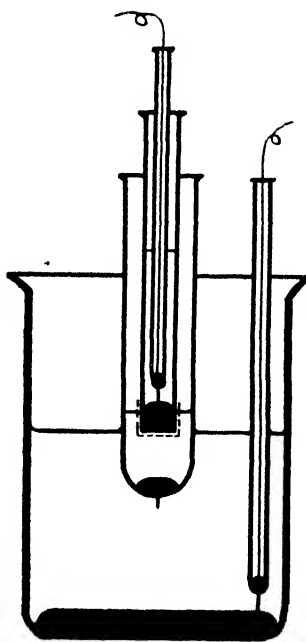
Fig. I.23.   Keys' Electro-dialysis Apparatus.

removed. In this way diffusable substances may be thoroughly removed from protein solutions.

Micro Electro-dialysis

A simple and very useful form of electro-dialysis apparatus (fig. I.23) is that of Keys,* which has been used for separating the alkali metals in serum from protein and other organic matter.

* Keys, A., *J. Biol. Chem.*. 1936, **114**, 450.

The cell consists of pure mercury electrodes separated by a semi-permeable membrane. On application of a current cations migrate to the cathode and amalgamate with the mercury there. Subsequently the amalgam is decomposed by shaking with acid, and the excess acid is then titrated with standard alkali.

The cathode vessel consists of a tube 1·5 cm. diameter and 10 cm. long. A cellophane strip, softened by soaking in distilled water, is forced in position over one end of the tube and maintained in a stretched position until dry. The edges are then trimmed and the membrane cemented to the tube with collodion.

About 20 g. of pure mercury are placed in the cathode vessel which also contains a standard acid.

The anode vessel consists of a test-tube about 2·5 cm. diameter and 10 cm. long, into the bottom of which is sealed a small piece of platinum wire. About 5 g. of mercury are placed in the test-tube.

The anode vessel is suspended in a small beaker, about 6·5 cm. tall, which contains diluted sulphuric acid and sufficient mercury to cover the base.

Contacts with a 120 volt battery are made through platinum wires which dip into the mercury (see fig. I.23).

The substance to be dialysed is placed in the anode vessel and a standard amount of acid is placed in the cathode vessel. The cathode vessel is lowered into the fluid in the anode vessel and a direct current of 110 volts is passed through the system. Bubbles will rise in the outer anode vessel until dialysis is completed. This process usually requires from 25–60 minutes.

The following examples illustrate the special value of this procedure:

(a) *Determination of "Total Bases" in Urine**

Reagents

1. $N/50$ Sulphuric acid.
2. $N/5$ Sodium hydroxide.
3. Mercury: this must be *purified* (see p. 443).

Method. 0·5 ml. of mercury is placed in the anode vessel of the apparatus together with about 20 ml. of water, and 0·2 ml. of serum or 0·1 ml. of urine are added from a Krogh pipette (p. 145). In the cathode vessel are placed 1·5 ml. of mercury and exactly 2·0 ml. of $N/50$ sulphuric acid. The cathode vessel is lowered into the fluid of the anode vessel, the electrodes are placed so as to make good contact with the mercury, and a current at 110 volts is passed through the apparatus.

When bubbles cease to rise in the outer anode vessel it can be taken that electro-dialysis is completed. The cathode vessel is then lifted out and the current is switched off. The electrode is rinsed with 1 ml. of distilled water

* Adair, G., and Keys, A., *J. Physiol.*, 1934, **81**, 162.

and the mercury-base amalgam is decomposed by agitation or passing air bubbles through. The excess acid in the cathode vessel is then titrated with $N/5$ alkali, using a Rehberg burette (p. 151) and 0·02% methyl red in 60% alcohol as indicator. During this process a current of air is used to mix the solution thoroughly (see fig. I.24).
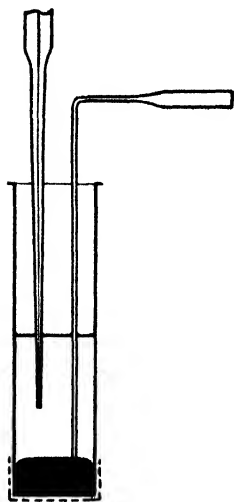
Fig. I.24.
Titration of Bases after Electro-dialysis.

(b) *Separation of Sodium and Potassium from Serum, Urine, etc.*[*][†]

Electro-dialysis can be used to separate sodium and other bases occurring in biological materials from interfering phosphates and proteins, prior to gravimetric estimation. In this way wet ashing is avoided.

The preliminary separation of bases by electro-dialysis can be followed by the precipitation of potassium with sodium cobaltinitrite and subsequent estimation by the ceric sulphate method in the cold, according to the technique of Kaye.[‡]

Ammonia is removed by a preliminary electrolysis using a platinum cathode.[§]

(c) *Estimation of Bone Minerals* [||]

Bone is brought into solution with acid and subjected to electro-dialysis. The metallic ions which collect at the cathode are dissolved off and estimated by the usual micro methods (see Parts III and IV).

* Sobel, A., Kraus, G., and Kramer, B., *J. Biol. Chem.*, 1941, **140**, 501.
† Sobel, A., Hancock, and Kramer, B., *J. Biol. Chem.*, 1942, **144**, 363.
‡ Kaye, I. A., *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 310.
§ Consolazio, W., and Talbot, J., *J. Biol. Chem.*, 1940, **132**, 753.
|| Sobel, A., Rochenmacher, M., and Kramer, B., *J. Biol. Chem.*, 1944, **152**, 255.

MICRO-CHEMICAL TECHNIQUE IN
REACTIONS INVOLVING PRECIPITATION

QUANTITATIVE precipitation is the simplest method of separating the components of mixtures, and it is frequently used as an initial purification process in volumetric or colorimetric analysis. Especial care must be taken to ensure that precipitates needed for exact analysis are deposited quantitatively from solution in a state of chemical purity. Essential precautions in precipitation analysis are therefore discussed below.

When a chemical reaction leads to the deposition of a precipitate, the quantity of material may be either in deficit or in excess of the "theoretical" or stoichiometric amount. Losses are due to (a) the partial solubility of the precipitate or to (b) delayed precipitation, which is equivalent to slow crystallisation from a super-saturated solution.

Excessive precipitation is due to the co-precipitation of an unwanted impurity or to adsorption upon the particles initially deposited. Troubles of both types lead to systematic errors in analysis, and once they have been recognised as such they can usually be compensated for by modifying the experimental technique by methods which are instanced below.

Losses due to the solubility of the precipitate usually occur when the precipitate is being washed with the pure solvent. Consequently the volume of wash solution should be kept to a minimum. Rather than washing with a single large volume of solvent it is advisable to use a succession of four, or more, small washings, and allow the precipitate thoroughly to drain between each washing. In such cases, too, it is useful to wash with a cold saturated solution of the precipitate. Thus in the micro-estimation of calcium (p. 168) the precipitate should be washed with a saturated solution of calcium oxalate rather than with distilled water. An alternative procedure is to use a solvent in which the precipitate is much less soluble than in the precipitating reagent (e.g. 95% alcohol in place of water).

Delayed precipitation and after-precipitation may be dealt with in a number of ways. Vigorous shaking of the solution, rubbing the sides of the vessel with a rubber "policeman" (compare p. 202) or with the *rounded* end of a glass rod, and the addition of a small quantity of alcohol, are typical of the practical methods of meeting this difficulty.

In micro-chemical work the time for complete precipitation must be studied carefully. Occasionally this may be shortened by using a *scavenger*, i.e. a substance with similar chemical reactions which is added in larger amount to the solution so that this, on precipitation, drags down the desired compound with it (compare Part IV, pp. 259–260). The separation of radium with barium is the classical instance of the use of this technique.

This procedure may be used with advantage if the substance to be estimated tends to form colloidal precipitates which are difficult to filter. Thus in the micro-estimation of traces of mercury, copper or arsenic salts can be added in some excess, and, when precipitated as the sulphide, these take down the small amount of colloidal mercuric sulphide also (see p. 262).

The temperature at which a reaction occurs often influences the completeness and purity of micro-precipitation. Thus in the estimation of lead as chromate the precipitation is complete and pure only if the reagents are added to the boiling lead-containing solution. Again, in the precipitation of barium sulphate, boiling of the solution prevents the co-precipitation of potassium salts, etc.

The pH of the solution very often influences the ease of precipitation, and often completeness and purity are only attainable between fine limits of pH. In these cases the use of buffer solutions is advisable.

The purity of a precipitate is sometimes influenced by the precipitation procedure. The manner of addition of the reagent, e.g. (i) rapidly in a squirt, (ii) drop by drop with shaking between additions (so that until precipitation is complete the reagent is not present in excess), (iii) slowly with even mixing, or (iv) with violent shaking after each addition of the reagent, can all affect significantly the success of the operation. Trial and error usually determine the best way to obtain reproducible results, and an analyst is well advised to refer to the original literature for hints as to the exact procedure.

Co-precipitation is a very complex source of trouble, and the modifying effect of ions other than of the substance to be estimated should always be borne in mind. In some cases, boiling the solution before, after, or during precipitation assists in avoiding this trouble, which is generally much less with crystalline granular precipitates than with gelatinous precipitates or very fine powders.

Often errors due to co-precipitation can be reduced greatly by redissolving the initial precipitate (after separation and washing) and then repeating the precipitation procedure. This is an operation which can be carried out quite speedily in centrifuge tubes.

Sometimes it is impossible to be certain that the constitution of a precipitate is in accordance with theory, no amount of re-solution and reprecipitation being successful in achieving this result. In such cases it is permissible to carry out a control estimation with a standard solution of the substance to be estimated, and therefrom to obtain a factor for the determination. An instance of this is afforded by the estimation of potassium by the cobaltinitrite method (p. 168). If this device is resorted to, then it is essential that the unknown solution should be treated down to the minutest detail exactly as was the standard solution.

NOTES ON SPECIAL ORGANIC REAGENTS
FOR THE ANALYSIS OF METALS

THERE are now available to analysts a number of synthetic organic compounds which have specific reactions with particular metallic cations, or which form pure, easily separable co-ordination compounds which are insoluble in water, but easily soluble in organic solvents such as chloroform. With the use of these reagents several highly specialised procedures for the detection and quantitative micro-analysis of individual metals have been developed in recent years.

F. Feigl's excellent monograph on "spot tests"* should be consulted for information concerning the use of these special reagents in qualitative micro-analysis, whilst the more recent monograph of E. B. Sandell, "Colorimetric Methods for the Determination of Traces of Metals,"† deals with considerations relevant to quantitative analysis.

Whilst the majority of these sensitive reactions are best used as colorimetric procedures, several of which are illustrated in Part IV, many can be adapted to micro-gravimetric or micro-volumetric analyses also (see Part III); and in view of the general applicability of these special reagents the salient points concerning their chemical properties are best considered in this first, more general, section of this book.

1. Dimethyl-glyoxime: CH_3—C——C—CH_3
$$\underset{NOH}{\overset{\|}{}} \quad \underset{NOH}{\overset{\|}{}}$$

This substance was the first specific organic reagent for a metallic cation to be introduced into general use in analytical chemistry. Tschugaev, in 1905, showed that in the presence of ammonia it would react with 1 part of nickel in 400,000 to give a red colour, and gave, with larger amounts, a weighable red precipitate of composition $[CH_3C(NOH)--C(CH_3)NO]_2Ni$, which is easily filterable and can safely be dried at 110°–120° C. Filterstick procedure (p. 21) is to be recommended for this gravimetric analysis on the micro-chemical scale.

Whilst other metals, such as copper or cobalt, also give red products with dimethyl-glyoxime, their colour reactions are very much less sensitive than that given by nickel, and by working in the presence of a tartrate or by precipitating the red nickel complex from a solution containing cyanide, it is easily possible to effect the quantitative gravimetric separation of nickel from other metals on both macro and micro-analytical scales.

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* Feigl, F., "Qualitative Analysis by Spot Tests" (revised edition), Elsevier Pub. Co., Amsterdam and New York, 1947.
† Interscience Publishers Inc., New York, 1945.

In 1924 F. Feigl (*Ber.*, 1924, **57**, 758) showed that the sensitivity of the colour reaction between alkaline solutions of nickel and dimethyl-glyoxime is increased at least fourfold by the addition of an oxidising agent, such as bromine-water, sodium hypochlorite, or hydrogen peroxide. A dark red solution is then produced, containing a compound of quadrivalent nickel—which, however, cannot be separated gravimetrically. The application of this reaction in colorimetry is given on p. 290.

2. 8-Hydroxy-quinoline, "Oxine":
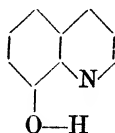
8-Hydroxyl-quinoline forms insoluble co-ordination compounds, $R^{\cdot}(C_9H_6ON)_n$, with a whole range of metals, such as aluminium, magnesium, and zinc. These products are very sparingly soluble in neutral aqueous solution, and so may be separated from excess of reagent by filtering or by centrifuging, and can, when pure, be weighed after drying at 130°–140° C. Whilst the micro-gravimetric analysis of oxine precipitates can safely be conducted by the filter-stick technique, micro-volumetric estimation, involving the dibromination of free 8-hydroxy-quinoline with bromide and bromate in acid solution, is both more rapid and more sensitive. This is illustrated for the analysis of magnesium on p. 175.

Some oxine precipitates give coloured solutions in chloroform which may be estimated colorimetrically. A more general and much more sensitive colorimetric procedure, however, is to dissolve the precipitate in acid alcohol, and then to couple up with diazotised sulphanilic acid to give an intensely coloured azo dyestuff; whilst a third method is to cause the oxine to reduce phospho-molybdo-tungstic acid to an intense blue product.

Separations of individual metallic ions as their oxinates cannot be effected as specifically as is possible with dithizone complexes (see below). Nevertheless, by careful pH adjustment, certain separations can be carried out quite successfully. Again, oxinate precipitation by undesired impurities can sometimes be prevented by complex ion formation. As an example of this, the separation of iron and magnesium may be instanced: if a solution containing both iron and magnesium is made alkaline with caustic soda and sodium tartrate is added until any precipitate redissolves, then on the subsequent addition of 8-hydroxy-quinoline in alcohol only the magnesium complex deposits. Again, iron may be separated from aluminium, since in the presence of acetic and malonic acids the iron complex, but not the aluminium compound, will precipitate.

However, 8-hydroxy-quinoline is best used as a quantitative precipitant for individual metals subsequent to their preliminary separation from others. It is most useful in the estimations of aluminium, calcium, magnesium, and zinc, and renders them amenable to micro-volumetric or colorimetric analysis.

3. Diphenylthiocarbazone, "Dithizone": C_6H_5—N=N—CS—NH—NH—C_6H_5

This sparingly soluble substance, which may be made by the interaction of phenylhydrazine and carbon disulphide followed by mild oxidation of the primary product,[*] is a particularly useful reagent for separating and estimating traces of certain heavy metals, with which it forms intensely coloured co-ordination compounds.

These complexes may be of two types: (a) thio-enolic products, derived from substitution of —SH groups, e.g.

$$
\begin{array}{ccc}
& C_6H_5 & \\
& N\!\!-\!\!-\!\!N & \\
M'\!-\!S\!-\!C & & M' \qquad \text{or} \\
& N\!\!=\!\!=\!\!N & \\
& C_6H_5 &
\end{array}
\qquad
\begin{array}{ccc}
& C_6H_5 & \\
& N\!\!-\!\!-\!\!N & \\
C\!-\!-\!S\!-\!-\!M'' & \\
& N\!\!=\!\!=\!\!N & \\
& C_6H_5 &
\end{array}
$$

(M′=monovalent metal.)   (M″=bivalent metal.)
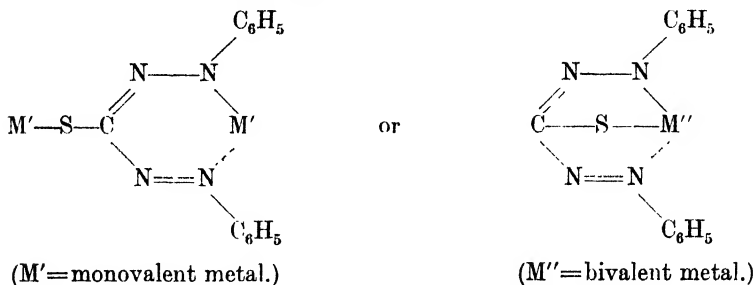
and (b) thio-ketonic types, derived from substitution of N—H groups:

$$
\begin{array}{ccc}
& C_6H_5 & \\
NH\!-\!N & \\
S\!=\!C & & M' \qquad \text{or} \\
& N\!\!=\!\!=\!\!N & \\
& C_6H_5 &
\end{array}
\qquad
\begin{array}{ccccc}
& C_6H_5 & & C_6H_5 & \\
NH\!-\!N & & N\!-\!-\!NH & \\
S\!=\!C & & M'' & & C\!=\!S \\
& N\!\!=\!\!N & & N\!\!=\!\!N & \\
& C_6H_5 & & C_6H_5 &
\end{array}
$$
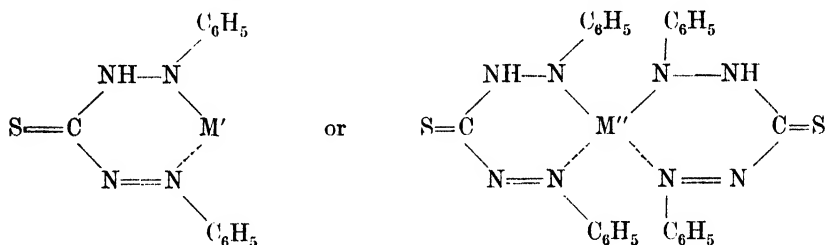
The keto form (b) is generally formed in neutral or faintly alkaline solution if the dithizone is not present in large excess. Acidification or addition of an excess of dithizone usually transforms a metallic complex to the keto form. Alternatively, alkalis tend to convert ketonic forms to enolic forms (a) should the latter exist. It will be noted that the thio-enolic complexes contain twice as much metal as do the ketonic complexes (b). However, few of these complexes have, as yet, been analysed accurately.

The following metals, which lie close together in the long series of the Periodic Table, form dithizone complexes:

Mn, Fe++, Co, Ni, Cu, Zn;
Pd, Ag, Cd, In, Sn++;
Pt, Au, Hg, Tl, Pb, Bi.

In analytical use, dithizone is dissolved in chloroform or a similar solvent and shaken with a very dilute aqueous solution of the metallic ion. The

[*] "Organic Syntheses," Vol. 25, p. 38 (Wiley, New York, 1945).

co-ordination complex then passes into the solvent layer as it is formed, and so may be separated. The efficiency of this process depends, however, upon the solubility product of the co-ordination complex in the aqueous layer. This may be represented as

$$M^+ + H—DT \rightleftharpoons M—DT + H^+$$

giving

$$[M—DT] = K . \frac{[M^+] . [H—DT]}{[H^+]}$$

and   *Extractibility of Complex* $= \dfrac{[M—DT_n] \text{ organic layer}}{[M^{n+}] \text{ water layer}}$

$$= \text{Const.} \frac{[H—DT]^n \text{ organic layer}}{[H^+]^n \text{ water layer}}$$

from which it can be seen that the production of a dithizone complex from any particular metallic cation can be controlled (i) by adjustment of the $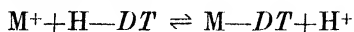p$H of the aqueous solution, and (ii) by the formation of water-soluble complex ions, e.g. complex cyanides, which may depress the ionic concentration of the free metallic cations M^{+n}. Thus, despite the fact that dithizone reacts to form water-insoluble complexes with so many metals, its reactions may be made almost specific for very many of them.

Thus if an aqueous solution be adjusted with acid to pH 1·0 or there-abouts, then only copper, mercury, palladium, and silver form solvent-extractable complexes, except in the presence of very large concentrations of some other metallic cations. Consequently, if the solvent layer is separated and then washed with acid of the appropriate pH, any inter-fering metals may be removed and a single pure product can be left for analysis. In this way traces of copper or mercury can be extracted from large amounts of lead.

If after the removal of the metals which combine with dithizone at pH 1, the acidity is lowered to about pH 3, then lead and bismuth will react, and may be extracted similarly. An alternative procedure for dithizone separation is to add the organic solution of the reagent to a faintly alkaline solution of the mixed metals and extract completely. Then on shaking the chloroform layer with acid of pH 3 the metals which combine only in alkaline conditions are passed into the aqueous layer. A further washing with acid of pH 1 will then separate lead and bismuth, leaving copper, mercury, etc., in the organic solvent.

In strongly alkaline solution (pH 12), when excess of citrate or tartrate has been added to prevent the precipitation of their hydroxides, cadmium, cobalt, nickel, copper, mercury, and the noble metals may be separated from all others.

Complex ion formation may be used even more specifically to effect the separation of dithizone co-ordination compounds. Thus in alkaline solution

in the presence of cyanide, lead, mercury, silver, and copper react to give dithizone complexes.   At pH 5·0 in the presence of sodium thiosulphate, lead, tin (stannous), cadmium, and zinc react; but if potassium cyanide is also added, then only tin and zinc give complexes.   Thiocyanate may also be used in this way.   In dilute acid solution it prevents the formation of complexes with all but mercury, gold, and copper ions, but if cyanide is present also, then only mercury and copper react.

The chief difficulty in the dithizone technique of separation of metals lies in the possibility of the formation of both ketonic and thio-enolic co-ordination compounds, which have different solubility products.   Only by the use of rigidly standardised procedures may errors due to this be prevented.

On account of the low solubility, even in organic solvents, and the intense greenish-blue colour of dithizone itself, it can be used only on the micro-chemical scale, and most rigid precautions have always to be taken to avoid chance contamination of the apparatus and of the water or other solvents used.

Several further practical points have to be considered if the manipulative procedure is to be made quantitatively accurate.   If an excess of free dithizone is left in the aqueous layer then there is a possibility of washing out some of the metallic complex from the chloroform layer when removing unwanted water-soluble substances.   A further solvent extraction of the wash water and reunion of this extract with the main solvent layer counteracts this error very largely.

Another difficulty is the tendency of dithizone to oxidise to diphenyl-thiocarbodiazone, C_6H_5—N=N—CS—N=N—C_6H_5, which is insoluble in both acid and alkali, but which gives an intensely coloured chloroform solution, which therefore interferes with the colorimetric estimation of metallic dithizone complexes.   All preparations of dithizone contain a certain amount of this oxidation product, and should therefore be purified, immediately before use, by the method given on p. 189.   The stock solution of dithizone in chloroform should be protected from light to prevent the occurrence of oxidation, and should be made up from solid material only when required.   In addition to other common oxidising agents, it should not be forgotten that iron and copper salts will oxidise dithizone in the presence of cyanide.   For this reason it is advantageous to add hydroxylamine hydrochloride to the reaction mixture to prevent progressive oxidation.

It has been claimed that di-α-naphthyl-thiocarbazone may be used in place of dithizone, and that it is more sensitive and gives brighter colours. As yet, however, this substance has not had any wide application in micro-analysis.

Volumetric applications of dithizone are given on pp. 188–190 and colorimetric applications on pp. 284–286.

4. β-Naphthyl-thioglycollic-amide, "Thionalide":

$$HS-CH_2-CO-NH-C_{10}H_7$$
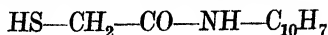
This substance is a fairly new reagent which has great potentialities for the colorimetric estimation of traces of metals.   In many ways it resembles hydrogen sulphide in its chemical properties, but is much more pleasant to work with, and can be used in solution in either alcohol or acetic acid. The precipitated thionalide of a metal may be separated by centrifuge technique (pp. 33–35), dissolved in N sulphuric acid and alcohol, and then made to react with phospho-tungstic-molybdic acid and formamide to give the well-known blue reduction compound which can then be measured colorimetrically.

In mineral acid solution copper, silver, gold, mercury, tin, arsenic, bismuth, platinum, and palladium form sparingly soluble precipitates from amongst the Group II metals.   Thionalide therefore serves as a means of separating cadmium, lead, and antimony from the other members of the sulphide group.   As little as 1 mg. in 10 ml. of solution of any of the above metals is usually detectable, although with arsenic as small a quantity as 0·1 mg. may be precipitated.

In the presence of a tartrate and excess of alkali, copper, gold, mercury, cadmium, and thallium can be precipitated, and in this way cadmium can be separated from lead.   In cyanide solutions containing tartrate, gold, thallium, tin, lead, antimony, and bismuth are precipitated, though if such a solution be made strongly alkaline then only thallium is precipitated. Thus by a judicious series of operations thionalide may be made a specific reagent for a number of Group II metals.

On account of its oxidisability, solutions of this reagent should be prepared just prior to use.

5. Sodium Diethyl-dithiocarbamate: $(C_2H_5)_2N-CS-S-Na$

This is another useful reagent which forms complex coloured thiols. With copper salts it yields a brown colour which is sensitive to 1 part in 50 million, and again the complex can be extracted from water with carbon tetrachloride.   This is of value in water analysis.   The reagent is not specific for copper.   Silver, lead, and mercury give white precipitates, whilst bismuth, iron, nickel, and maganese all give coloured compounds.

As an alternative to sodium diethyl-dithiocarbamate, one may use diethylammonium diethyl-dithiocarbamate, which can easily be prepared *in situ* from diethylamine and carbon disulphide.   The scope of this reagent for the estimation of traces of metals in foods, etc., is indicated on p. 385.

By suitably adjusting the conditions, dithiocarbamate reagents may be made quite specific for copper.   Thus in the presence of $2N$ hydrochloric acid, copper, bismuth, mercury, the noble metals, and trivalent arsenic may be extracted from aqueous solution into chloroform.   If this extract is evaporated and oxidised then bismuth can be removed with dithizone

(see p. 285), the more noble metals can be reduced with formaldehyde, and copper can be extracted selectively from the residue with dithiocarbamate.

Again, dithiocarbamates are most useful for separating trivalent arsenic compounds from pentavalent arsenic compounds, since the latter do not pass into the chloroform layer from an aqueous solution containing $2N$ hydrochloric acid. If it is desired to remove the pentavalent arsenic from the acid solution, after separation of the trivalent arsenic, then reduction with potassium iodide and bisulphite followed by a second dithiocarbamate extraction is all that is necessary.

6. α-Nitroso-β-naphthol:
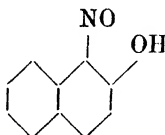
This is useful as a specific reagent for the determination of cobalt in solutions from which metals of the preceding analytical groups have been removed. The red precipitate, which can easily be collected and weighed, is the cobaltic salt, $Co(C_{10}H_6O_2N)_3$. In analysis, therefore, the cobalt solution must be treated with caustic soda and hydrogen peroxide to give cobaltic hydroxide, which can then be dissolved in acetic acid and treated with the nitroso-β-naphthol reagent. Micro-gravimetric analysis by filter-stick technique is quite feasible; a colorimetric process is given on p. 291.

With this same reagent palladium also gives an insoluble product, $Pd(C_{10}H_6O_2N)_2$, suitable for gravimetric analysis.

7. Alizarin Dyestuffs

Many alizarin dyestuffs give highly insoluble lakes with metals such as aluminium, thorium, and zirconium (amongst others). These are rarely suitable for gravimetric micro-analysis on account of their gelatinous nature, but in extreme dilution they often yield colloidal dispersions suitable for colorimetry (see p. 307). Again, the production of these highly-coloured lakes can often be prevented by complex ion formation, and this action can be made the basis of a number of volumetric processes (compare p. 141), amongst which the estimation of fluoride is undoubtedly the most important (p. 191).

8. p-Dimethylamino-benzylidene-rhodanine:
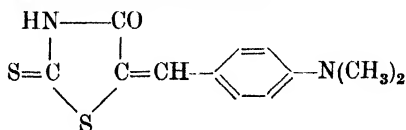
This reagent was introduced by Feigl (Z. Anal. Chem., 1928, **74,** 380) for the detection of silver, with which, in weakly acid solution, it gives a red-violet colour, suitable for colorimetric estimation. Similar colours are

given by mercury, gold, platinum, palladium, and cuprous ions, but the reaction can be made specific for silver by first adding potassium cyanide and then acidifying with $2N$ nitric acid. Whereas $KAg(CN)_2$ is not stable in acid and gives silver ions, mercuric cyanide is not dissociated, whilst the complex cyanides of the other noble metals are stable in acid solution.

Mercury, however, can be estimated colorimetrically with this reagent by working in $0.05N$ nitric acid, though under these conditions many anions, such as sulphate and nitrate, interfere, and it is advisable to effect a preliminary separation of mercury by electro-deposition (p. 494).

TABLE OF
MICRO-GRAVIMETRIC METHODS FOR ANALYSIS OF METALS

Element	Reagent and Preparation	Method of Collecting and Drying Precipitate	Factor and Formula of Precipitate	Reference
Aluminium.	8-hydroxyquinoline; 5% solution in 10% acetic acid. Filter before use.	Filter-stick or tube. Dry at 140° C.	$F = 0.05871$. $Al(C_9H_6ON)_3$.	Benedetti-Pichler, *Mikrochem.*, *Pregl Festschrift*, 1929, **6**.
Antimony.	(i) A solution of ethylenediamine and chromium chloride forms a complex with antimony sulphide.	Filter-stick or tube.	$Cr[NH_2(CH_2)_2NH_2]$. SbS_4.	Spacu-Pap, *Z. Anal. Chem.*, 1938, **111**, 254.
	(ii) Dissolve approx. five times the theoretical amount of pyrogallol in 10 ml. of air-free H_2O and add this to the solution for analysis. (In dilute acid solution the reagent is selective.)	Filter-stick or tube. Wash precipitate several times with water. Dry to constant weight at 105° C.	$F = 0.46321$. $Sb(C_6H_5O_4)$.	Vogel, A. I., "Text-book of Quantitative Inorganic Analysis," 1942, p. 519.
Beryllium.	1% solution of tannin. Separate Al, Co, Fe^{III}, Th, Zr, Ti by precipitation with tannin in acid solution. Beryllium comes down when filtrate is made slightly alkaline.	Crucible. Ignite carefully, add drop of nitric acid, reheat and weigh as BeO.	$F = 0.36051$ as BeO.	Vogel, A. I., "Text-book of Quantitative Inorganic Analysis," 1942, p 537.
Bismuth.	2% solution of pyrogallol. Bismuth should first be separated, e.g. with dithizone from copper, lead, antimony, iron, etc. (see p. 46).	Filter-stick or tube. Dry at 110° C.	$F = 0.62937$. $Bi(C_6H_3O_3)$.	Feigl, F., and Ordett, H., *Z. Anal.Chem.*, 1925, **65**, 448.
Cadmium.	1 g. quinaldinic acid is neutralised with caustic soda solution and diluted with water to 30 ml.	Filter-stick or tube. Wash with cold water and dry at 125° C.	$F = 0.24612$. $Cd(C_{10}H_6NO_2)_2$.	Ray, P., and Bose, M. K., *Z. Anal.Chem.*, 1933, **95**, 400.
Calcium.	1% solution of picrolonic acid.	Filter-stick or tube. Wash with ice-cold water, and dry to constant weight in stream of dry air.	$F = 0.05642$. $Ca(C_{10}H_7O_5)_2\ 8H_2O$.	Kisser, J., *Mikrochem.*, 1923, **1**, 25; Bworzak, R , and Reich-Rohrwig, *Z. Anal. Chem.*, 1931, **86**, 98.
Cobalt.	0·1 g. α-nitroso-β-naphthol dissolved in 50 ml. of 50% acetic acid.	Crucible. Wash with water. Dry at 110° C. and ignite to Co_3O_4.	$F = 0.09640$. $Co(C_{10}H_6O_2N)_3\ 2H_2O$.	Hecht, F., and Korkisch, F., *Mikro-Chim. Acta.*, 1938, **3**, 313.

TABLE OF

MICRO-GRAVIMETRIC METHODS FOR ANALYSIS OF METALS—Continued

Element	Reagent and Preparation	Method of Collecting and Drying Precipitate	Factor and Formula of Precipitate	Reference
Copper.	1 g. of salicylaldoxime is dissolved in 5 ml. of alcohol and diluted to 100 ml. with water.	Filter-stick or tube.	$F = 0.1894$. $Cu(C_7H_6O_2N)_2$.	Reif, W., *Mikrochem.*, 1931, **9**, 424.
Iron.	1% solution of Cupferron: prepare freshly as the solution decomposes on standing. Heating also causes decomposition. In strong acid solution only Ti and Zi interfere.	Crucible. Precipitate washed with dilute HCl, then with $5N$ ammonia to convert to ferric hydroxide, and the crucible is ignited. Weigh as Fe_2O_3.	$F = 0.6990$ as Fe_2O_3.	"Organic Reagents for Metals," Hopkins and Williams, London, 1943, p. 19.
Lead.	1% solution of picrolonic acid (separate first with dithizone, see p. 190). Precipitate in neutral solution.	Filter-stick or tube. Solution is cooled to 0° C. before filtering off complex, which is washed with ice-cold water and dried at 140° C.	$F = 0.2725$. $Pb(C_{10}H_7N_4O_5)_2 1.5H_2O$	Hecht, F., Reich-Rohrwig, Brantner, H., *Z. Anal. Chem.*, 1933, **95**, 152.
Magnesium.	2% solution of 8-hydroxyquinoline in $N/1$ acetic acid. Preliminary separation by group methods is necessary.	Filter-stick or tube. The precipitated complex is filtered whilst hot, after standing for 15 minutes, then dried at 100°–105° C., yielding the dihydrate after washing with 1% ammonia. (A trace of sodium taurocholate aids transference to the filter.)	$F = 0.0698$. $Mg(C_9H_6ON)_2 2H_2O$.	Strebinger, R., and Reif, W., *Mikrochem.*, *Pregl Festschrift*, 1929, 319. Hough, W., and Ficklen, J., *J. Am. Chem. Soc.*, 1930, **52**, 4752; Creuss-Callaghan, G., *Biochem.*, 1935, **29**, 1081.
Mercury.	5% solution of copper ethylenediamine nitrate and 2% solution of potassium iodide.	Filter-stick or tube. The Hg complex is washed with water, alcohol, and finally ether, and dried in a vacuum desiccator.	$F = 0.22488$. $Cu(C_4H_{16}N_4)(HgI_4)$	Vogel, A., "Text-book of Quantitative Inorganic Analysis," 1942, p. 505.

TABLE OF

MICRO-GRAVIMETRIC METHODS FOR ANALYSIS OF METALS—Continued

Element	Reagent and Preparation	Method of Collecting and Drying Precipitate	Factor and Formula of Precipitate	Reference
Molybdenum.	Dissolve 1 g. of 8-hydroxyquinoline in a minimum of acetic acid. Dilute to 100 ml., add dilute ammonia dropwise until a slight permanent ppt. separates and then add more acetic acid until the latter dissolves.	Filter-stick or tube. Hot solution is filtered and the complex is washed with hot water; dry to constant weight at 130°–140° C.	F=0·23051. $MoO_2(C_9H_6ON)_2$.	Fleck, H. R., and Ward, A. M., *Analyst*, 1933, **38**, 338.
Nickel.	0·5% solution of dimethylglyoxime in alcohol.	Filter-stick or tube. Complex is washed with hot water and dried at 115° C.	F = 0·2032. $Ni(C_4H_7O_2N_2)_2$.	Armit, W., and Marden, A., *Proc. Roy. Soc.*, 1906, **77B**, 420; Pollak, I., *Mikrochem.*, 1924, **2**, 17.
Palladium.	0·5% solution of dimethylglyoxime (nitrate must be absent). (Gold and platinum interfere.)	Filter-stick or tube (or crucible if complex is ignited and weighed as metal). The complex can be dried at 100°–110° C.	F=0·3167. $Pd(C_4H_7O_2N_2)_2$. F=as Pd.	Zschiegnee, H., *Ind. Eng. Chem.*, 1925, **17**, 294.
Potassium.	5% solution of sodium-6-chloro-5-nitrotoluene-3-sulphonate (ammonia and barium must be absent). The method has only half the sensitivity of the cobaltinitrite method (see p. 168).	Filter-tube or stick. Minimum of solution used; concentrate on the water-bath if necessary; cool to below 5° C. before filtering; wash complex with a few ml. of ice-cold water and dry to constant weight at 120° C.	F=0·1350. $C_7H_5NO_2ClKSO_3$.	Davies, H., and Davies, W., *J. Chem. Soc.*, 1923, **123**, 2976.
Sodium.	Reagents: (i) Uranyl acetate 1 g., 30% acetic acid 0·6 g., water to 6·5 g. (ii) Zinc acetate 3 g., 30% acetic acid 0·3 g., water to 6·5 g. These solutions are mixed at 20° C., left for several hours, and filtered.	Filter-stick or tube. Solution for analysis must be concentrated to 1–2 ml. Precipitate is washed four times with 1 ml. of reagent, then five times with alcohol saturated with sodium zinc uranyl acetate in 1 ml. portions, and finally with ether, and dried at 40° C.	F=0·1495. $NaZn(UO_2)_3$, $(CH_3COO)_9, 6H_2O$.	Barber, H. M., and Kolthoff, I. M., *J. Am. Chem. Soc.*, 1928, **50**, 1625.

TABLE OF

MICRO-GRAVIMETRIC METHODS FOR ANALYSIS OF METALS—Continued

Element	Reagent and Preparation	Method of Collecting and Drying Precipitate	Factor and Formula of Precipitate	Reference
Tin.	10% solution of Cupferron. Remove Cu, Pb, As^{III}, and Sb by precipitation with H_2S in 1% HF solution. Then make strongly acid with 1% HCl, 1% H_3BO_3, and 1% H_2SO_4 before adding the Cupferron.	Crucible. Filter complex after crushing with a glass rod and washing with cold water. Dry and ignite carefully to constant weight and weigh as SnO_2.	$F = 0.78766$ as SnO_2.	Vogel, A., "Text-book of Quantitative Inorganic Analysis," 1942, p. 521; Mack, M., and Hecht, F., *Mikro-Chim. Acta.*, 1937, **2**, 227.
Titanium.	1% solution of Cupferron. (Iron may be first removed as sulphide in alkaline tartrate solution.)	Crucible. Precipitate is washed with dilute HCl containing a little Cupferron, dried and ignited to constant weight as TiO_2.	$F = 0.600$ as TiO_2.	"Inorganic Reagents for Metals," Hopkin and Williams, Ltd., London, 1943, p. 20.
Tungsten.	1% solution of oxine in alcohol. pH range of precipitate is between 4·05 and 5·65.	Filter-stick or tube. Wash precipitate with hot water, dry to constant weight at 120° C.	$F = 0.36477$. $WO_2(C_9H_6ON)_2$.	Fleck, H. R., *Analyst*, 1937, **62**, 378.
Uranium.	Oxine solution (as prepared for molybdenum). Precipitate in slightly acid solution.	Filter-stick or tube. Wash with hot then cold water and dry to constant weight at 110° C.	$F = 0.33840$. $UO_2(C_9H_6ON)_2$, C_9H_7ON	Kroupa, E., *Mikro-Chim. Acta.*, 1938, **3**, 306; *Mikrochem.*, 1939, **27**, 1.
Zinc.	1% solution of oxine. Mg does not co-precipitate if pH is adjusted to 4·6.	Filter-stick or tube. The precipitate is washed with hot water and dried at 160° C.	$F = 0.1902$. $Zn(C_9H_6ON)_2$	Cimerman, C., Frank, D., and Wenger, P., *Mikrochem.*, 1938, **24**, 149.

For fuller details of the above procedures the appropriate literature should be consulted. A bibliography, together with various procedures for the determination of metals using organic reagents, is contained in Hopkin and Williams' "Text-book of Organic Reagents for Metals." Also the macroprocedures described in A. I. Vogel's "Text-book of Quantitative Inorganic Analysis" (1942), if applied to the single elements in solution can be adapted to micro-procedures under the appropriate conditions.

PART II

MICRO-ANALYSIS OF ORGANIC COMPOUNDS

By G. Ingram and W. A. Waters

PART II

MICRO-ANALYSIS OF ORGANIC COMPOUNDS

GENERAL INTRODUCTION

THE credit for the initiative in developing accurate milligram-scale methods for the ultimate analysis of organic compounds must be given to the late Professor F. Pregl, of Graz in Austria, who justly became world-famous not only as an experimenter but also as a teacher of quantitative micro-chemistry.

Pregl's micro-chemical methods, which were developed from the standard combustion techniques of Liebig and Dumas, depend essentially upon the use of the Kuhlmann micro-balance (described on pp. 11–15), but their great success is due to his careful, systematic study of experimental errors and of the ways in which they could be eliminated. His original monograph on the subject, "Die quantitative organischen Mikroanalyse,"* which gives a clear account of the subject, should be read by everyone who wishes to understand the underlying principles of micro-chemical technique, though at the present time substantial improvements have been made in his original methods and several other excellent accounts of the subject have been published.†

At the present time it can be stated unhesitatingly that the micro-chemical methods for the ultimate analysis of organic compounds are so accurate that they can be recommended for general use in preference to the older methods of macro-analysis. They reduce the time required for analysis to at least one-fifth, and, more important, permit one to handle rare compounds of which a small fraction of a gram only is available.

In laboratories which do not possess an accurate micro-balance, milligram-scale analysis cannot, unfortunately, be carried out. Semi-micro-analysis, requiring 10–50 mg. of material, can, however, be conducted with the use of any good analytical balance sensitive to 0·01 mg., provided that the operational technique of micro-analysis is adopted. It has, indeed, been recommended widely for the training of students.‡

The experimental methods described below for analysis on the milligram scale can all be used, without modification, for larger quantities of material, and hence no special treatment of semi-micro methods is called for.

* An English translation, published in 1924, is available.
† E.g. Roth's revisions of Pregl's monograph (pub. 1937 and 1947); Niederl and Niederl, "Micromethods of Quantitative Organic Elementary Analysis," New York, 1938.
‡ Cf. Gattermann-Wieland, "Laboratory Methods of Organic Chemistry," translation by McCartney, London, 1938. Niederl and Niederl, "Micromethods of Quantitative Organic Elementary Analysis," New York, 1938.

Some of the original micro-chemical methods were only applicable to restricted classes of organic compounds. The difficulties caused by the occasional presence of elements such as chlorine, sulphur, or arsenic have now been overcome, and consequently in the following pages will be described only tested procedures safely applicable to organic molecules of all types.

It must be emphasised, however, that an entirely different technique of micro-analysis is required for the estimation of "trace elements" in organic material—e.g. for the determination of iodine in biological tissue, in which case one has to estimate a few μg. of the element in as much as 100 g. of substance. Special volumetric procedures for the determination of "trace elements" in organic material are described in Part III of this volume (pp. 166–218), whilst colorimetric procedures are described in Part IV (pp. 277–403). Polarographic methods of analysis (Part V, pp. 434–483) are also proving to be of great value in this field of micro-chemistry.

Again, in the field of biological chemistry, Van Slyke and his colleagues have developed many useful gasometric methods of organic analysis which often prove to be applicable in other fields of chemistry. Thus for the analysis of blood, serum, and similar colloidal material, he devised a method of estimation of carbon involving a quantitative oxidation to carbon dioxide, the volume or the pressure of which was then measured. These and similar biochemical procedures, which are described in Part VI of this volume, can on occasion be resorted to with advantage, and deserve wider recognition by the average organic chemist.

ESTIMATION OF CARBON AND HYDROGEN BY COMBUSTION

Principles

In the classical Liebig method of combustion analysis the organic substance, contained in a porcelain boat, is heated in a slow oxygen or air stream until there is no residue, and the oxidation of the carbonaceous matter is completed by passing the hot gases over a column of red-hot copper oxide made from copper wire. Water is trapped and weighed in sulphuric acid or calcium chloride, and carbon dioxide is collected in strong caustic potash solution. If elements other than C, H, O are present, then the procedure must be modified. Nitrogenous compounds are burnt in a *limited* supply of air, usually after admixture with powdered copper oxide, and a freshly reduced spiral of copper gauze has to be placed after the copper oxide column to reduce acid-forming oxides of nitrogen (NO, NO_2).

If halogens are present, then a silver gauze spiral must also be placed before the reduced copper spiral to retain the halogens in the form of involatile silver halides. Nevertheless, the volatilisation of copper halides in the hot part of the tube soon causes the packing to block, and also leads to rapid deterioration of the glass of the combustion tube.

The element sulphur causes still more trouble. To prevent the passage of the acidic oxides SO_2 and SO_3, a tube packing of granular lead chromate has to be used; this is rather prone to melt and then either to block the tube or to cause it to crack upon cooling.

For the combustion of many substances the packed tube filling can be dispensed with by following Dennstedt's procedure and using a large excess of oxygen, and either catalytic "contact strips" of platinum foil or a very hot tube* to complete the oxidation of organic vapours (including CH_4, CO, etc.), but again modifications have to be made for retaining the halogens and the oxides of nitrogen or sulphur.

All difficulties in combustion analysis become proportionally more serious on the micro-chemical scale. Nevertheless, Pregl produced a satisfactory "universal filling" which could be used for the estimation of carbon and hydrogen in organic compounds of all types.

This is illustrated in fig. II.1. In the main, the combustion is completed at red heat (600°–700° C.) by a column (ii) of mixed copper oxide wire and lead chromate granules, retained between short asbestos plugs. The lead chromate serves to retain sulphur, arsenic, etc. Halogens are retained by plugs (i), (iii) of silver-wool, of which the outermost (i) can be removed and

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* Belcher and Spooner, *J. Chem. Soc.*, 1943, 313.

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renewed without disturbing all the rest of the tube filling.   Finally, oxides of nitrogen are decomposed catalytically by passing the gases through granular lead peroxide (v) maintained at 170°–200° C. by surrounding that portion of the tube with a jacketted vessel containing boiling cymene or decalin.

In addition, Pregl standardised his combustion procedure by maintaining a constant rate of flow of gases through the tube, thus minimising the chance of errors due to irregular burning, inflammation, or explosion of the organic substance.   To this end he used (a) a constant head of pressure of inflowing oxygen, and (b) a constant suction at the outflow, securing the latter by attaching a Mariotte bottle to the end of the absorption train (compare fig. II.15).   Further, he adjusted the gas pressure inside the tube by the use of a choking plug (iv) of ignited fibrous Gooch asbestos, placed so that the hot gases were under a slight positive pressure until they had passed through the main oxidation filling (ii).

The back-diffusion of incompletely burnt vapours is not serious on the micro-chemical scale, since the empty entrant end of the combustion tube contains a very large excess of hot oxygen.   Even this slight risk can be further minimised by inserting, behind the boat containing the substance,
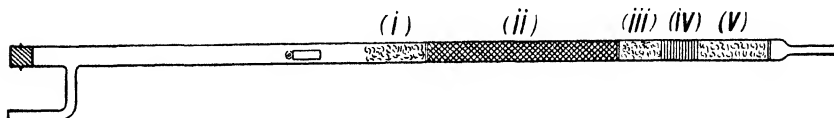
Fig. II.1 (about ⅕ scale).   Combustion Tube with Pregl's Universal Filling.

a glass or quartz rod baffle so as to increase the forward velocity of the entrant oxygen stream.

Tubes packed with Pregl's "universal filling" give most satisfactory results, and under favourable circumstances can serve for about 200 combustions.   By the time that refilling becomes necessary on account of partial blockage of the packing it usually happens that the tube itself must be discarded, consequent upon the development of cracks or pinhole leaks at points at which the lead or silver packing has sintered into the glass.   This trouble is particularly serious if genuine Supremax glass cannot be obtained and more brittle glass or fused quartz tubes have to be used as alternatives. However, this difficulty can be overcome by using *boat fillings** so that the heavy metals do not come in contact with the combustion tube itself.

One well-tested "universal boat filling" is depicted in fig. II.4.*   Granules of silver vanadate, maintained at 600°–650° C., serve both to complete oxidation and to retain halogen and sulphur compounds, whilst a second boat, filled with granular lead peroxide, maintained at 195° C., effects the final destruction of oxides of nitrogen.   Exhaustion of the silver vanadate is indicated by a distinct colour change from yellow to reddish-brown.

* Ingram, G., *J. Soc. Chem. Ind.*, 1943, **62**, 175.

If organo-metallic compounds are being analysed, then the life of the filling is considerably increased by the insertion of an initial plug of copper oxide gauze or, alternatively, of the additional boat filling containing ceric oxide which is described on p. 66.

When boat fillings are used a positive oxygen pressure can be maintained throughout the whole apparatus, and by controlling the supply of oxygen by means of a constant-head flowmeter, suitably designed to guard against any "blow-back" consequent upon an explosion in the tube, hand regulation of the gas flow can be obviated.

Oxygen used in combustion analysis must be freed from both carbon dioxide and moisture. The use of a separate gas holder is not essential, for an oxygen stream from a cylinder, metered through a needle-valve, can be purified and used directly.

Since traces of organic vapours are often evolved from rubber, it is advisable to use ground-glass joints for all connections between the purifier and the combustion tube. These should be lubricated with phosphoric acid, not grease.

Absorption Apparatus

High accuracy in micro-chemical analysis is obtainable only by the use of small but efficient absorption apparatus light enough to be weighed on a micro-balance. Pregl used straight, thin-walled glass tubes of 8–9 mm. diameter and 9–10 cm. long, with 3·3–3·5 mm. bore capillary tube ends, and fillings of granular calcium chloride and soda-lime. Constancy in weight, after blank tests, to within 0·01 mg. can be secured if the standard wiping procedure, described on pp. 16–17, is rigorously carried out.

Absorption-tubes fitted with ground-glass stoppers possess many advantages over tubes of the Pregl pattern. In particular, they can be weighed with an internal oxygen atmosphere; this obviates the necessity of sweeping out the whole apparatus with a stream of dry air at the end of each analysis. Tubes of a very convenient pattern have been designed by Blumer (fig. II.2, *V* and *W*). They are about 14 cm. long and weigh about 10 g. when filled. To prevent adventitious loss of weight upon wiping, consequent upon extrusion of tap grease from the ground-glass stoppers, it is now customary to cut a circular channel near the top of each stopper to act as a grease trap, and to lubricate only the lower portion of the tube and stopper.

All connections between absorption-tubes must be made by glass-to-glass joins inside 2·5 cm. long pieces of vaseline-impregnated, aged, thick-walled rubber tubing. Similar rubber tubing blocked with glass rod should be used to seal the ends of Pregl absorption-tubes when they are not in use. This use of *impregnated* rubber tubing is essential; it prevents loss of carbon dioxide by diffusion through rubber, which otherwise is a serious source of error. To prepare it, pressure tubing should be boiled out with alcohol and

water, dried, and then immersed in molten vaseline or soft paraffin wax inside a flask which is evacuated until no more bubbles of air are evolved from the rubber; on readmission of air vaseline fills the pores of the rubber, where it congeals on cooling. Suitable impregnated tubing can, however, be purchased from most laboratory dealers.

Very efficient absorbents for both water and carbon dioxide can now be purchased in convenient granular form. For the retention of water by far the most efficient reagent is anhydrous magnesium perchlorate, known as "anhydrone." Since it is a neutral, stable salt it has no tendency to retain carbon dioxide (compare average granular calcium chloride). On the micro-chemical scale, a solid packing of granular soda-lime is the most effective absorbent for carbon dioxide, for a single tube packing can be used repeatedly before it is exhausted. Several excellent types of porous granules, which often incorporate fibrous asbestos, are on the market under trade-names such as Ascarite, Sofnolite, etc. Usually these also contain an indicator which changes colour as the carbon dioxide neutralises the free alkali. Tubes packed with Ascarite, etc., should also contain a layer of anhydrone to prevent any loss of water vapour.

A guard tube, also filled with anhydrone granules, should always be attached to the end of the combustion train to prevent the back-diffusion of moisture from the laboratory air.

Combustion Furnaces

In setting up apparatus for organic micro-analysis, the most important requirement is a good combustion furnace. Both gas and electrically heated designs are now available. In certain recent models automatic control of the combustion has been achieved by the use of a clock-controlled movable furnace.*

Several workers prefer gas-heated furnaces, since with a flame local heating is obtained almost instantly and the temperature is easily controlled. On the other hand, uniform heating of a length of combustion tubing is difficult and there is inevitably an escape of much heat and of fumes into the laboratory. The latter drawback can be serious, since a micro-chemical laboratory should be kept at constant temperature and humidity. The compilers therefore prefer to use electrical heating whenever possible. Heating elements can easily be constructed in any laboratory, but many excellently designed micro-combustion furnaces giving rheostat or thermo-couple temperature control are now on the market. Attention is directed to the split type of furnace, in which the heating elements are built into two parts so arranged that the combustion tube can be cooled rapidly, if needed, by pushing back the heating elements on their mounting frame without disturbing the rest of the apparatus. The independent motion of at least one furnace along the axis of the combustion tube is essential.

* Reihlen, W., *Mikrochem.*, 1937, **22**, 285.

A Standard Combustion Apparatus*

1. *Main Assembly* (fig. II.2). The furnaces, constant-head flowmeter, pre-heater, control units, and switches, together with an ammeter, are mounted on an all-metal base with a central metal support, containing at the rear two compartments, one for the electrical control units and the other for the flowmeter and pre-heater. A sliding support S is provided for the absorption-tubes.

The combustion tube, mounted at a fixed height, is heated by three easily adjustable furnaces, M, A, B.

2. *The Heating Mortar M*, of conventional Pregl design, is a hollow Pyrex glass cylinder, electrically heated, lagged with asbestos and mounted in a metal cylinder with removable end-plates. The hollow cylinder, to which is fused a reflux air condenser some 40 cm. long, is 7 cm. in length and 3·5 cm.
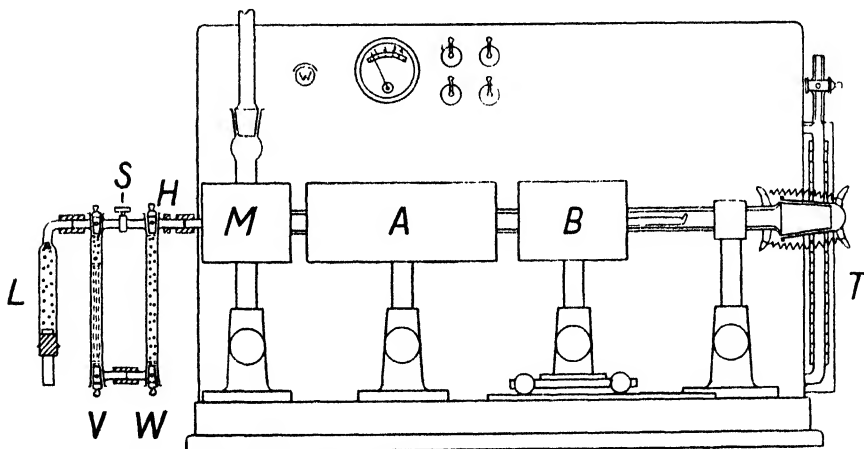
Fig. II.2. Apparatus for Estimation of Carbon and Hydrogen by Combustion.

in external diameter. The concentric annular space, through which is passed the combustion tube, is 1·7 cm. in diameter. The glass vessel is filled with dekalin, which boils at 195° C.

3. *The Main Heating Furnace A* is an electrically wound cylinder 15 cm. long. It is regulated to give a temperature of 600°–650° C., and heats the portion of the combustion tube which contains the silver vanadate filling.

4. *The Movable Furnace B*, also cylindrical, is 9 cm. long. It is attached by an adjustable support to a metal base, which has fitted at its corners four small grooved wheels which run on rails fixed to the main base-plate in the area through which the furnace has to be moved during the combustion.

5. *Oxygen Supply*. Cylinder oxygen, regulated by a Pregl precision cock which is situated behind the indicator scale, passes through the

* Ingram, G., *J. Soc. Chem. Ind.*, 1942, **61**, 112; cf. ibid., 1939, **58**, 34.

flowmeter (see below) at constant pressure and is purified in the pre-heater and drying train (fig. II.3). It enters the combustion tube via the side-arm (K, fig. II.4) by way of a detachable glass tube with spring-fitted ground-glass joints.

6. *The Flowmeter.* A design due to Ingram* for automatic control of the oxygen supply is shown in fig. II.3. The guard tubes C, G, the wide bulb D, and the baffle plate P together provide adequate safeguards against blockage of the adjustable capillary tube C_1, through which the gas is metered, in the event of a sudden pressure change such as that which might result from an explosion of the substance in the combustion tube.

The oxygen flow rate should be adjusted to 6 ml. per minute by regulating the Pregl precision cock, and if the water level in the manometer tube
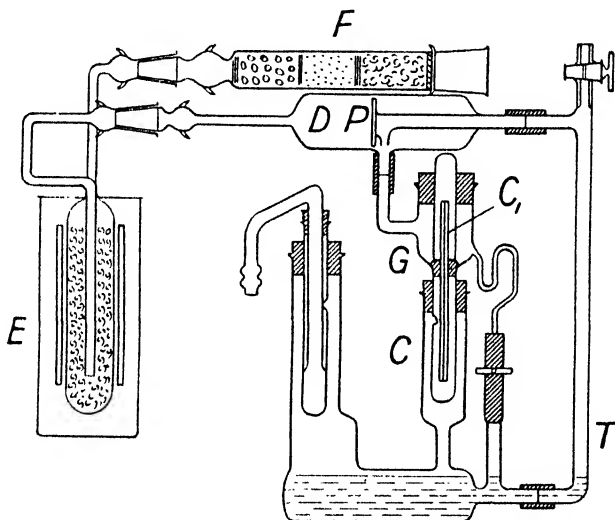
Fig. II.3 ($\frac{1}{5}$ scale).   Flowmeter, Pre-heater, and Drying Tube for Oxygen Supply.

(T, fig. II.3) is brought to constant level when commencing each series of combustions, no further regulation is necessary.

Much simpler types of capillary tube flowmeters and pressure regulators can, of course, be used,† though most of these necessitate frequent hand adjustment of the flow rate. It is always advisable to incorporate on the outflow side of the capillary a wide bulb for damping out sudden pressure variations.

7. *The Pre-heater* (E, fig. II.3) consists of a Pyrex tube about 12 cm. long and 2·5 cm. diameter. The inlet tube dips almost to the bottom, so that the oxygen is heated before coming into contact with the catalyst filling, which is a 1 : 1 mixture of cerium dioxide and vanadium pentoxide

* *J. Soc. Chem. Ind.*, 1942, **61**, 113.
† Cf. Sucharda and Bobranski, *Chem. Ztg.*, 1927, **51**, 568; *Rocz. Chem.*, 1928, **8**, 209.

supported on pumice. Bulb E, which is heated electrically, is regulated so that a temperature of 300°–350° C. is maintained. It is connected by a B14 joint to the drying tube F, which is filled successively with layers of coarse granular calcium chloride, anhydrone, and finally Ascarite.

8. *The Combustion tube* (XY, fig. II.4) should be made either of Supremax glass or of clear fused silica. It is 1·2–1·3 cm. bore and 55 cm. long, tapering at one end to a beak 3 cm. long and 2–3 mm. diameter. A bent side-arm K is sealed on 5 cm. from the other end. This has a ground A14 socket-joint for attachment to the purified oxygen supply. The wide mouth X of the combustion tube is ground to a standard B19 cone, and is closed by a spring-fitted B19 socket-cap of glass.

On assembling the apparatus, a small amount of anhydrone, held between cotton-wool plugs, is placed in the side-arm to remove any moisture possibly arising from the phosphoric acid which should be used to lubricate all the ground joints in the apparatus.

If silica combustion tubes are used it will be found that they gradually become translucent, especially in the region of the tube where the carbon is burnt away. Translucent silica tubes are apt to become porous to gases
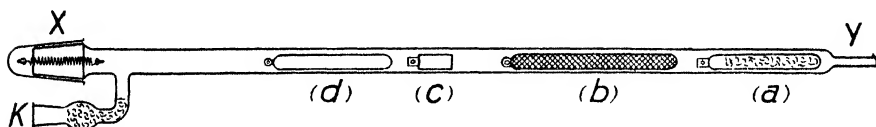
Fig. II.4 ($\frac{1}{5}$ scale). Combustion Tube with Ingram's Boat Filling.

when hot. Fortunately it is not a complicated operation to cut out this portion of the tube and seal on a new length of clear silica.

9. *Absorption tubes.* The use of absorption tubes of Blumer's pattern (fig. II.2) is advised. The tube W placed nearest to the combustion furnace is filled with anhydrone, held in place by cotton-wool plugs, of which one should be marked with ink to distinguish between the inlet and the outlet of the gas stream. The other tube V, for carbon dioxide absorption, is filled at the outlet end with a 3 cm. layer of anhydrone, whilst the remaining space is filled with Ascarite granules held in place by cotton-wool plugs.

When in use the anhydrone tube W is always attached directly to the beak end Y of the combustion tube by a glass-to-glass join inside a short (2 cm.) piece of impregnated pressure tubing (p. 61). Around the inlet tube H should be placed the hooked end of a piece of stout copper wire, projecting from the heating mortar M. By conduction of heat along this wire to the glass tube, the premature condensation of moisture is avoided.

If by mischance the tubes are connected the wrong way round during a combustion, then they must be cleaned out and refilled before proceeding with any other analysis.

M.Q.M.A.—6

A guard tube L filled with a 6 cm. layer of anhydrone should always be placed after the carbon dioxide absorption tube. This guard tube should be attached directly to the combustion tube when the apparatus is not in use.

Boat Fillings for Carbon and Hydrogen Analysis (see fig. II.4)

When boat fillings are used, the active material should be prepared in the form of porous granules of 10–14 mesh. The porcelain boats should fit snugly into the combustion tube. Before use they should be boiled with aqua regia, washed with distilled water, dried, and then ignited strongly.

Lead peroxide granules (*a*), prepared according to Pregl,* should be placed at the end of the tube in a boat 7–8 cm. long. Each filling, which will weigh about 2·5 g., will last for about 15 determinations before slightly high results will be obtained with nitrogenous compounds.

Silver vanadate granules (*b*), in a boat 10 cm. long, should form the main contact material. To prepare them, dissolve 18 g. of vanadium pentoxide in $2N$ caustic soda solution, filter, and add an excess of silver nitrate solution. Boil up the mixture and then filter off and dry the yellow precipitate of silver vanadate.

To make porous granules, stir 6 g. of 10–14-mesh pumice-stone into a crucible containing a thick paste prepared from 5 g. of silver vanadate and a little water. Heat, with continual stirring, over a fairly strong flame till all the water has been driven off, and then continue the heating, with stirring, until the salt melts and a soft, even mass is obtained. Finally, ignite the product strongly for 4 hours to ensure that all volatile products have been removed.

A single boat filling should last for about 150 combustions; a change in colour indicates the necessity for refilling (see p. 60).

For the combustion of organo-metallic compounds it is advantageous to place, before the silver vanadate, a small porcelain boat containing the following mixture: 4 g. of cerium oxide, 10 g. of litharge, 6 g. of silver dichromate, and 3 g. of silver oxide are ground together and then fused in a crucible. 4 g. of lead chromate granules are stirred into the fused mass, and the stirring is continued as the mixture cools so that a granular product is obtained. This should be sieved before use.

The above mixture may be used as an alternative to silver vanadate as the main oxidation catalyst.†

Methods of Weighing out Substances for Analysis

Solids. Weigh non-metallic compounds into freshly ignited platinum boats; weigh compounds containing metallic elements into porcelain boats

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* Pregl, F., "Quantitative Organic Micro-analysis," 1924, p. 24.
† Ingram, G., *J. Soc. Chem. Ind.*, 1942, **61**, 114.

which have previously been cleaned with chromic/sulphuric acid mixture and ignited.

To ensure that the ultimate residue is free from carbonate, mix substances containing sodium or potassium, after weighing, with powdered potassium dichromate, using for this a short length of platinum wire which should be left in the boat during the combustion.

Viscous substances should also be weighed in boats, the transference being made by means of a clean, thin glass rod.

Liquids. Weigh liquids in glass capillary tubes made by drawing out quill tubing to about 2 mm. bore. Prepare a fresh tube for each analysis by melting in the middle a 10 cm. length of this capillary tube till there forms a blob of glass which can be drawn out and cut off to form a solid handle with a rounded end (fig. II.5, *a*). Tap a small crystal of potassium chlorate into the sealed end of the prepared capillary tube and fix it in position by melting. During the combustion the potassium chlorate will evolve oxygen and thereby ensure the complete expulsion of the liquid from the tube. Finally, soften the capillary tube in a flame, applied about 2 cm. away from the potassium chlorate crystal, and draw out so as to give a fine hair capillary tube about 2–3 cm. long (fig. II.5, *b*).

After wiping it with chamois-leather, weigh accurately, and fill the capillary tube by the

Fig. II.5 (⅔ scale).

(*a*) and (*b*) Capillaries for handling liquid.
(*c*) Weighing-bottle.

evacuation technique described on p. 28. Before reweighing, wipe the tube externally with cotton-wool and then chamois-leather.

Liquids of low vapour pressure can be filled into open-ended tubes, but with liquids of high vapour pressure the end of the hair capillary must be sealed before weighing the substance. When carrying out the combustion, place the weighed tube inside a sleeve made of platinum or resistance glass so that the fine hair capillary faces the oxidation filling.

A similar technique should be used in analysing liquids for nitrogen (p. 76), sulphur, or halogens (p. 86) by combustion methods. In estimations of halogens or sulphur, however, ammonium nitrate should replace potassium chlorate.

Weigh hygroscopic substances, before and after drying, inside a small stoppered weighing-bottle (fig. II.5, *c*), made of thin-walled glass. This should be kept inside the case of the micro-balance.

Combustion Procedure

1. *Assembly and Testing.* Before first assembling the apparatus, clean out the glass parts with sulphuric/chromic acid mixture, wash them thoroughly with distilled water, and dry them in an oven. After connecting up the apparatus and filling the purification train (fig. II.2), insert the two boat fillings into the combustion tube, switch on the heating furnaces (A, B, M), regulate them to the required temperatures, and bake out the tube for 2 hours. Then, combust in the usual way about 5 mg. of an organic substance*; after a further half-hour of heating carry out a check analysis with a known compound. If this does not yield a satisfactory result, continue the baking-out process for another hour before making another test.

2. *Normal Combustion Procedure:*

(i) Connect the absorption-tubes to the combustion tube and leave them for 10 minutes to fill with oxygen. Meanwhile weigh out the sample in a platinum boat (see p. 66).

(ii) Disconnect the absorption-tubes, wipe them by the standard procedure (p. 16), and weigh. Reconnect them and then push the weighed substance C into the combustion tube to within 5 cm. of the large furnace A, following it by the baffle rod d (fig. II.4).

(iii) Place the movable furnace B at the end of its track, just behind the sample boat, switch on the current in this circuit, and open the taps of the absorption-tubes. After about 5 minutes roll the movable furnace forward gradually so that after 10–20 minutes it reaches the main furnace A and all the substance has been distilled over into the oxidation material.

(iv) After a further 20 minutes close the outlet tap of the Ascarite tube V. Wait until the level of the liquid in the flowmeter tube T has fallen to the zero mark, then close the remaining taps, disconnect the absorption-tubes, and remove them for cooling, wiping, and weighing. Finally, switch off the current in the movable furnace B. The next sample for analysis may be weighed out during the above sweeping out period, and for speed in operation one should have duplicate sets of absorption-tubes.

3. *General Routine.* When commencing a series of combustion analyses, first bake out the combustion tube in oxygen for 1 hour, in which time the temperature from the combustion furnaces A and M should have become steady. Then make the check analysis with a pure compound, and until this is satisfactory do not commence the analysis of unknown materials. If possible, place for sequence in analysis compounds of similar chemical type. Results accurate to within 0·1% are obtainable in this way.

The main furnaces A, M should not be turned off in between analyses, for otherwise another baking-out process will be necessary.

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* This operation brings to an equilibrium state the absorption of moisture by the lead peroxide granules (see Pregl, ibid., p. 31). Suitable substances are succinic acid or phenacetin, of pure M.A.R. quality.

THE DETERMINATION OF NITROGEN

Two general methods of determining the nitrogen content of organic substances are now available to the micro-analyst; both of these have been scaled down from well-known macro-chemical procedures.

Dumas' method of combusting a substance with copper oxide and collecting the resulting nitrogen gas over caustic potash is still the most reliable method of ultimate analysis, and except when dealing with materials which contain only a trace of nitrogenous matter, is to be recommended for organic molecules of all types, especially when the accuracy of the analysis is more important than the time taken to obtain the result. The whole procedure takes about 1 hour.

Kjeldahl's volumetric method of estimating nitrogen as ammonia by decomposing organic matter with hot concentrated sulphuric acid has now been developed on the micro-chemical scale to such a degree that it may be used with confidence with nitrogenous compounds of most types, excepting esters of nitric and nitrous acids. It is of especial value for the analysis of biological material, such as blood, protein, etc., and is by far the most convenient method to use when long series of similar analyses have to be performed. As little as 0·1–0·2 mg. of nitrogen can be estimated by the standard procedure given in this Part of the volume; still smaller quantities of material can be handled by the special biochemical procedures included in Part III (pp. 197–200 and 205–207).

Lacourt's method[*] of heating the substance with thoria-activated nickel in a hydrogen stream at 250° C. and so eliminating nitrogen as ammonia is another variant of the principle of volumetric estimation of nitrogen. As routine procedure it can be made quite convenient for mass analysis.

A. The Determination of Nitrogen by Combustion

The micro-chemical technique for determination of nitrogen by combustion has been developed by scaling down the standard Dumas' apparatus. When working with a micro-nitrometer graduated to 0·01 ml., one should plan to collect about 1 ml. of gas. This volume is given by about 5 mg. of a substance containing 20% of nitrogen. By combusting correspondingly larger quantities of material, accurate results can be obtained with substances containing down to 2% of nitrogen.

A very few compounds (e.g. some pyrimidines, purines, and pyrrole derivatives such as porphyrins) form a nitrogenous charcoal when heated with copper oxide, and consequently yield low nitrogen figures. This

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[*] Lacourt, A. *Bull. Soc. Chim. Belgique*, 1940, **49**, 167.

difficulty can be overcome by using an additional oxidising agent. Pregl*
and others have recommended that substances of these types should be
mixed with powdered potassium chlorate. Lead dichromate can also be
used† but unfortunately this slowly attacks the combustion tubes, and copper
acetate has been suggested as a preferable alternative.‡

Combustion Apparatus

1. *General Design*

Fig. II.6 illustrates a modern form of the micro Dumas apparatus, due
essentially to Reihlen and Weinbrenner.§

Purified carbon dioxide from a generator (see below, pp. 72–74) is
passed through a regulating tap and the copper inlet tube I to the
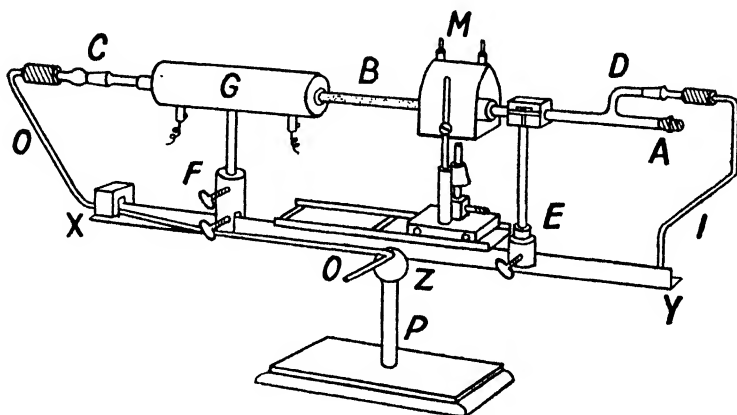
Fig. II.6.   Apparatus for Determination of Nitrogen by Combustion.

combustion tube ABC by means of the side-arm D. From C the gaseous
products pass through the narrow copper tube O to a nitrometer, where the
carbon dioxide is absorbed and the resulting nitrogen measured. The
combustion tube and the heating furnaces are mounted firmly, by supporting
rods and the slotted brass collars E, F, on a strip of angle-iron XY, which
is held at its middle point Z in a horizontal axle-bearing at the top of the
stout pillar P, which is about 9 in. high and forms a rigid part of the main
base-plate. By releasing the screwed joint at Z the combustion tube can
be tipped from a horizontal to an inclined position, for filling or emptying,
without disturbing the setting of the heating furnaces.

The narrow copper pipes I and O are secured to the frame XY with clips.
Their outer ends, mounted coaxially with Z, have ground B10 cone-joints
and fit into glass socket-joints on the leads from the carbon dioxide generator

* "Quantitative Organic Micro-analysis," 1930 edn., p. 94.
† Pregl-Roth, "Quantitative Organic Micro-analysis," 1937 edn., p. 85.
‡ Hayman and Adler, *Ind. Eng. Chem.* (Anal. Edn.), 1937, **9**, 197.
§ *Mikrochem.*, 1934, **23**, 285.

and to the nitrometer respectively, so that the tilting of the combustion tube need not cause any gas leaks.

The combustion tube $C-D$ and the copper leads I and O are joined by short pieces of thick-walled, paraffin-impregnated pressure tubing, which can be permanently sealed on by means of picein wax.

In comparison with Pregl's original linearly designed apparatus, with furnaces fixed at bench level, the compact set-up of fig. II.6 facilitates rapid batch analysis, since it is unnecessary to cool the whole of the combustion tube or remove it from its gas-tight settings at the end of each analysis.

2. *The Heating Furnaces*

The main, fixed, furnace G, which covers about 20 cm. of the combustion tube, should be adjusted to give a temperature of 600° C., and need not be turned off at the end of each analysis. For this, electrical heating has proved to be most convenient, and a simple heater can easily be made by winding nichrome wire on a silica tube of 16 mm. internal diameter, lagging well with asbestos, and enclosing in a brass tube with metal end-plates.

The movable furnace M, used to combust the substance, must be turned off at the end of each analysis, and before another substance can be analysed the end AB of the combustion tube must be allowed to cool nearly to room temperature. A cylindrical electric furnace is unsuitable for M on account of the very slow cooling due to the mass of the lagging, but either a movable split-type of electric furnace (p. 62) or a gas-heated furnace can be used.

Gas heating can be arranged quite easily by mounting a Bunsen burner on a base-plate about 3 in. square, having at its corners four small grooved wheels ($\frac{1}{2}$ in. diam.) which run on fixed rails (in this case, clamped to XY). The gas burner should be provided with a screw to regulate its height, and with a metal draught-screen to which is attached a brass tube which acts as a flame-spreader.

3. *Micro-nitrometers*

Accurate micro-nitrometers are made by many laboratory furnishers. They should have a capacity of 1·5–2·0 ml., be graduated to 0·01 ml., and be readable to 0·001 ml. with a lens. The gas inlet tube, sealed to the lower bulb at about 4 cm. from the bottom, should *not* protrude inside the nitrometer. This inlet tube should be of 1·0–1·5 mm. bore, so that fine gas bubbles are produced. It should enter the nitrometer horizontally, but its other end should bend upwards to a regulating stopcock B (fig. II.7), which has a glass barrel into which two grooves have been cut, in opposite directions at right-angles to the bore, so that a fine adjustment of the rate of gas flow can be obtained. Beyond the stopcock the capillary tube should be extended, with suitable bending, to end in a

spring-fitted B10 socket-joint which fits into the copper outlet tube O of the combustion apparatus.

The junction between the levelling bulb and the graduated nitrometer must be made with thick-walled rubber tubing which has been boiled in 10% caustic soda and then steamed out for an hour. This procedure both ages the tubing and helps to prevent the formation of foam-producing substances by the gradual attack of the strong caustic potash on the inside of the tubing.

4. *Carbon Dioxide Generators*

Most micro-analysts have hitherto used a Kipp's apparatus for preparing their air-free carbon dioxide. This is still the most reliable apparatus for intermittent use, but when several analyses have to be performed consecutively, as in a routing laboratory, it is much simpler to make use of a block of solid carbon dioxide ("drikold" or "cardice") which evaporates slowly but steadily at room temperature, giving pure carbon dioxide gas.

The precautions required in the manipulation of generators of both types are described below.

(*a*) *Air-free Carbon Dioxide from Drikold.* The simple generator designed by Hershberg and Wellwood* (fig. II.8) consists of a litre vacuum flask
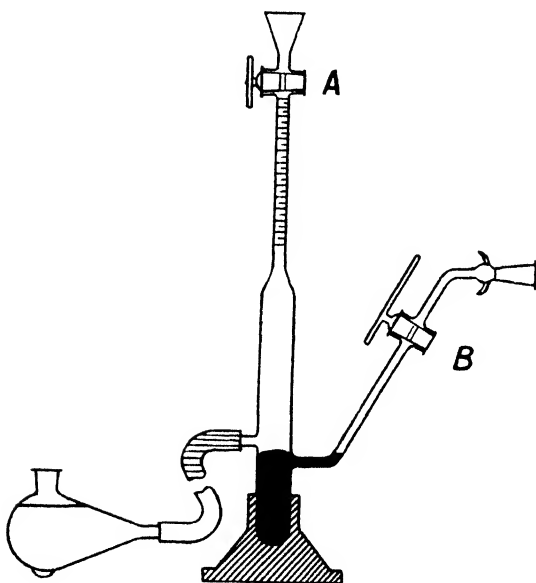
Fig. II.7.   Micro-nitrometer.

fitted with a rubber stopper carrying a thick-walled T-piece, to one limb of which is sealed a stopcock A for connecting to the combustion tube, and to the other a safety-valve B. This is a U-tube manometer, about 20 cm. long with a vent C placed half-way down the closed limb. At the vent there is fitted, between rubber washers, a diaphragm of hardened filter-paper, of porosity such that gas, but not mercury, can pass through.

A few hours before use the manometer is filled with clean, dry mercury to just over the top of the diaphragm and large lumps of drikold are placed in the flask. As the carbon dioxide evaporates it sweeps all the air from

* Hershberg and Wellwood, *Ind. Eng. Chem.* (Anal. Edn.), 1937, **9**, 303.

the flask and soon gives gas pure enough for micro Dumas' analysis. One lb. of drikold should last for about 5 days.

(b) *Generation of Air-free Carbon Dioxide in a Kipp's Apparatus.* Pregl has shown that the following precautions have to be taken in order to obtain carbon dioxide pure enough for micro Dumas' analysis:

(i) DESIGN. A large Kipp, of not less than 2 l. capacity, is required. The tap for connecting to the combustion apparatus should be fitted to the Kipp by means of a *wide* ground-in joint through which the generator can easily be charged with marble chippings; after assembly this joint should be secured with picein wax.

An auxiliary generator must be connected to the top bulb of the Kipp in order to prevent any air from dissolving in the main hydrochloric acid reagent and thereby penetrating into the main carbon dioxide stream. This auxiliary generator can simply be made from a filter-flask A, containing hydrochloric acid, into the neck of which is fitted the long stem of a separating funnel B (fig. II.9).

The wide part of B is charged with marble chippings, and is joined to the Kipp by the gas-tight lead C. The generator works automatically, since acid is sucked up by the drop in pressure in the top bulb of the Kipp when the latter is functioning, whilst any excess of carbon dioxide escapes through the side-arm of the filter-flask.

(ii) CHARGING. Marble chips, of the purest quality obtainable, are well washed with a dilute solution of hydrochloric acid, covered with water in a large beaker,

Fig. II.8. Hershberg and Wellwood's Carbon Dioxide Generator.

and boiled rapidly for 10 minutes. When almost cool, the chips, together with some of the water, are transferred to a filter-flask and a further quantity of hydrochloric acid is added. When the brisk reaction has subsided, the flask is corked and the side-arm is connected to a suction pump. By repeated shaking the remaining traces of air and carbon dioxide are removed. Suction is maintained until no more bubbles rise from the chips and the water is quite cold. The vacuum is then released slowly so that the pores of the marble become filled with calcium chloride solution. This marble is then filled into the central chamber of the main Kipp generator and a 3 : 1 mixture of concentrated hydrochloric acid and boiled water is poured in to fill the lower bulb and one-third of the top bulb.

The apparatus must then be de-aerated by dropping a few pieces of marble into the top of the Kipp to remove air from the upper bulb. It is then flushed out two or three times by opening the tap fully until a good evolution of gas takes place. The auxiliary generator is then attached and the assembly is left for two or three hours with the tap sufficiently open to produce a slow stream of gas (about one bubble per second).

After this preliminary treatment the Kipp should be connected to the combustion apparatus, the combustion procedure carried out in blank, and the gases issuing at the nitrometer examined carefully. Only "micro-bubbles" should be produced. These should be absorbed almost completely in the lower, wide part of the nitrometer, and the residues, which should ascend uniformly to the top of the graduated column, should not exceed 0·2 mm. in diameter. If the bubbles are not small enough, then the de-aeration of the Kipp must be repeated.

As a rule, a newly charged Kipp supplies sufficiently pure carbon dioxide only after two or three days' standing, since time is required for the air adsorbed on the surface of the glass to be given up to the carbon dioxide atmosphere.
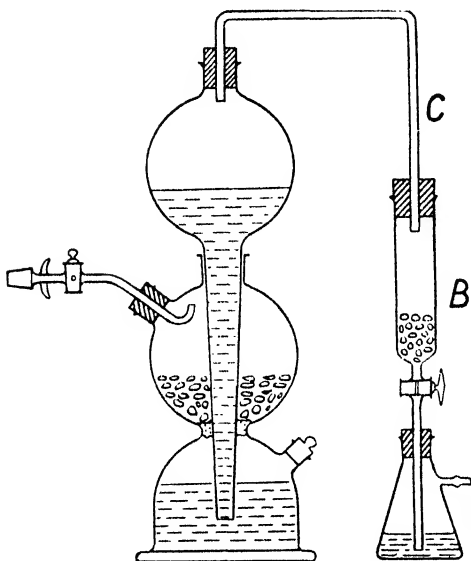
Fig. II.9. Production of Air-free Carbon Dioxide in a Kipp's Apparatus.

5. *The Combustion Tube and its Filling*

The combustion tube (fig. II.10), made of transparent quartz or preferably of Supremax glass, should be 60 cm. long and 1·0 cm. internal diameter. The exit end *C* has a narrow beak or an A10 ground joint for connection to the nitrometer, whilst a side-arm *D*, bent at right-angles and fitted with an A10 ground socket-joint, is sealed in at about 3 cm. from end *A*. The use of translucent quartz tubes is inadvisable, since after repeated use their interiors flake off and the tubes then become sufficiently porous to admit air when hot.

The combustion filling consists of (i) a 10 cm. layer of coarse, wire-form, copper oxide (see Note 1), which should protrude about 2 cm. beyond the furnace at the exit end. This is followed by (ii), a 6 cm. layer of reduced copper, prepared by heating the wire-form copper oxide in a stream of pure hydrogen; and (iii) by a further 12 cm. of coarse copper oxide. About

5 cm. of the latter should protrude beyond the fixed furnace G (Fig. II.6). Each successive layer of filling should be held in place by a pad of ignited asbestos about 2–3 mm. in thickness. The substance for analysis, mixed with fine copper oxide (iv) (see Note 2) should occupy about 12 cm. of the tube, and should be followed, at end A, by a quartz rod baffle 12 cm. long (v). A small plug of cotton-wool is placed inside the side-arm D to trap any acid spray from the Kipp.

NOTE 1.—The coarse copper oxide, in wire form, should be washed with 10% acetic acid, then with distilled water, dried in an oven, and heated strongly before use.

NOTE 2.—The fine copper oxide for mixing with the substance should be prepared from the above by grinding to a coarse powder under 10% acetic acid, and then washing and drying as given above. Precipitated copper oxide should not be used, since it produces too much resistance inside the combustion tube.
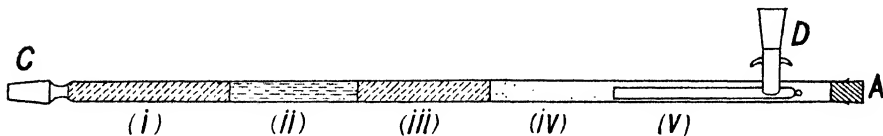
Fig. II.10. Filling of Combustion Tube for Estimation of Nitrogen.

Analytical Procedure

1. Testing of Apparatus

(i) The filled combustion tube is fitted into the furnace and the carbon dioxide generator is connected on and secured with springs. The furnace G is switched on, and for a period of 2 hours a stream of carbon dioxide is passed through the tube to remove adsorbed air from the copper oxide filling.

(ii) The nitrometer is prepared by cleaning thoroughly, greasing the stopcocks with vacuum-tap lubricant, pouring clean mercury into the nitrometer until its level is 5 mm. above the inlet tube, and finally filling the levelling bulb with filtered 50% caustic potash solution. This is prepared by dissolving 200 g. of A.R. potassium hydroxide pellets in 200 ml. of freshly distilled, carbon dioxide free water. After 24 hours the solution is filtered through sintered glass into a rubber-stoppered bottle.

(iii) After the 2 hours of preliminary heating, the nitrometer is attached to the combustion tube and, after allowing a further 5 minutes for sweeping air out of the inlet pipe, the lower stopcock B of the nitrometer is closed, and by raising the levelling bulb above the upper stopcock A, the nitrometer is filled with caustic potash solution. Stopcock A is then closed, leaving a trace of potash above the cock, and, after lowering the levelling bulb, the lower stopcock B is opened *slowly* until gas passes through at the rate

of one bubble per second.   Only micro-bubbles (see p. 74) should ascend the nitrometer.   If this is not the case, then the whole apparatus should be scrutinised carefully, and left, with a slow stream of carbon dioxide passing through it, for another hour before retesting.

2. *Weighing Out the Substance for Analysis*

(a) *Solids.* A platinum boat is heated, cooled on a copper block (p. 17), transferred to the balance pan, and counterpoised to the nearest milligram. It is then returned to the block, filled with 3–5 mg. of the powdered substance, brushed externally, and returned to the balance pan for exact weighing.

The boat is then held in platinum-tipped forceps, and its contents are transferred to a "mixing tube" made from an 8 cm. length of micro-Carius tubing.   The boat can be emptied completely by rotating it with one hand whilst gently tapping the mixing tube with the other.   After making sure that none of the substance is sticking to the outside, the boat is then reweighed.

(b) *Hygroscopic Substances.* These must be weighed out in porcelain boats, using a stoppered weighing bottle (Fig. II.5, c), and transferred directly to the combustion tube, in which they should be packed in fine copper oxide.

(c) *Liquids.* Liquids are weighed in capillary tubes containing potassium chlorate (see p. 67), and are wrapped in copper oxide gauze before insertion into the combustion tube.   These tubes should have so small a capacity that the final volume of nitrogen is not significantly affected by the volume of air which they enclose.   Viscous oils may be weighed into porcelain boats.

3. *Charging the Combustion Tube*

(i) The nitrometer is removed from the apparatus, clamping screw Z (fig. II.6) is loosened, and the forward end of the bar XY is dropped to bench level so that the combustion tube is in the filling position.

(ii) The "mixing-tube" is two-thirds filled with fine copper oxide, which is added through a bent funnel with a long neck.   It is then stoppered with a *rubber* stopper and the substance and copper oxide are uniformly mixed by thorough shaking.

(iii) The bent funnel is then put into end A of the combustion tube and the contents of the mixing-tube are loaded in.   The mixing-tube is rinsed out twice with fine copper oxide, which is added until the column in the combustion tube is about 12 cm. long.   The combustion tube is then refixed in the horizontal position, the quartz baffle is inserted, and end A is closed with a rubber stopper, after opening the tap of the carbon dioxide generator.

(iv) A stream of carbon dioxide is now passed through the tube.   After 5 minutes the nitrometer is attached, the bubble rate is adjusted to 1 per second, and after a further 5 minutes a test for the absence of air (see

Section 1 above) is made. Tests can be made at short intervals, and as soon as micro-bubbles only form, the combustion can be commenced.

4. *The Combustion*

(i) When the tube is air-free, the stream of carbon dioxide is cut off, the lower stopcock *B* of the nitrometer is opened fully, and the movable heater *M* is placed below the baffle and put on. When the stream of bubbles in the nitrometer has subsided, any froth at the top of the stem is expelled through tap *A*, and burner *M* is pushed slowly forward, thereby distilling the substance slowly through the filling of the combustion tube.

The time required for the combustion must be controlled by watching the rate at which gas bubbles enter the nitrometer; this must not exceed 1 bubble per second. Whenever gas ceases to enter the nitrometer, the furnace *M* may be pushed forward a little. The combustion usually takes from 15–25 minutes.

(ii) Stopcock *B* is then closed temporarily, the carbon dioxide supply is turned on, and the remaining nitrogen is driven over into the nitrometer by regulating stopcock *B* to give the standard bubble rate. At the same time movable furnace *M* is turned to full heat, pushed back to the baffle, and again advanced slowly up to the fixed furnace *G*.

(iii) When all the nitrogen has been driven over into the nitrometer, stopcock *B* is closed, furnace *M* is turned off, and the levelling bulb is raised and clamped at the same level as the caustic potash solution inside the nitrometer. The volume of nitrogen may be read off 15 minutes later. Any gas bubbles remaining on the meniscus of the caustic potash solution or on the mercury surface can be broken up by striking the rubber tubing with the edge of the hand. At the same time, one should record both the barometric height and the temperature, using an accurate thermometer which should be hung permanently near the neck of the nitrometer.

(iv) As soon as end *AB* of the combustion tube has cooled down, it is permissible to depress the tube at end *A*, remove the stopper, baffle, and fine copper oxide filling, and immediately proceed to the recharging of the tube for another analysis.

5. *Calculation of Results*

After measuring the nitrogen volume, correction must be made for the vapour pressure of the solution, retention of liquid near the top of the nitrometer, and for the possibility of contamination by traces of carbon monoxide and nitrous oxide.

Pregl,* as a result of intensive investigation, found that these errors could all be compensated for by subtracting 2% from the measured volume of nitrogen. The result is then corrected to N.T.P. and computed as a

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* Pregl, "Quantitative Micro-analysis," 1930 edn., p. 73.

weight percentage using the theoretical relationship: 22·4 l. of nitrogen at N.T.P. weigh 28·01 g.*

B. Volumetric Method of Nitrogen Determination

Principles

Kjeldahl's method of determining the nitrogen content of organic matter by (i) decomposition with hot concentrated sulphuric acid, (ii) liberation of the resultant ammonia with alkali and distillation into standard acid, and (iii) back titration with alkali, was originally intended for use with amino- and amido- compounds only.

This sequence of operations is satisfactory for the analysis of all biological material, but for the analysis of nitro, azo, or diazo compounds, such as dyestuffs or their intermediates, it is necessary to carry out an additional reduction process, though sometimes it is sufficient to add glucose to the digestion mixture.†
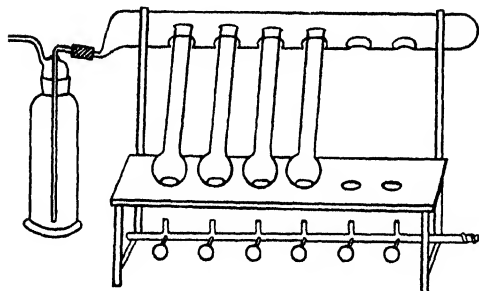
Fig. II.11.
Kjeldahl Flasks in Rack with Fume Collector.

When conducting Kjeldahl determinations on the micro-chemical scale it is essential to add a catalyst, such as selenium and mercuric sulphate,‡ to the sulphuric acid to ensure that the destruction of organic matter is complete. This procedure also speeds up considerably the overall time required for the analysis.

The ultimate accuracy depends upon the sensitivity of the final micro-titration, but even when it is advisable to make use of the ultra-micro technique described in Part III (pp. 197–200 and 205–207) the preceding stages of the analysis should still be carried out by the standard methods given below.

1. *Digestion with Concentrated Sulphuric Acid*

On the milligram scale the decomposition of organic matter is carried out in micro-Kjeldahl flasks, made from thick Pyrex or Jena glass tubing 1·5 cm. in diameter and 16 cm. long, having a bulb of about 15 ml. capacity. If a series of analyses have to be carried out, a stand such as that illustrated in fig. II.11 should be used. Here each flask is heated with its own

* Niederl and Niederl ("Micro-methods of Quantitative Organic Elementary Analysis") and other workers have suggested more complicated methods of evaluating the corrected volume of gas, but Pregl's empirical deduction of 2% gives results which are correct to well within the overall accuracy of the whole Dumas' analysis.
    † Elek and Sobotka, *J.A.C.S.*, 1926, **48**, 501.
    ‡ Belcher and Godbert, *J. Soc. Chem. Ind.*, 1941, **55**, 196.

micro-burner, and the mouth of each flask is fitted inside a glass flue-duct from which all fumes are sucked away to a water-pump in which they are harmlessly dissolved. The acid digestion must in all cases be carried out inside a fume-cupboard.

Solids for analysis should be weighed out in long-handled weighing-tubes (p. 17). Viscous materials should be weighed into small porcelain boats, which are then placed directly inside the Kjeldahl flasks; whilst liquids should be weighed out in capillary tubes, which should then be crushed inside the Kjeldahl flasks. Physiological liquids, such as blood, should be measured in from precision pipettes (such as the Krogh pipette, p. 145) or from calibrated tubes (such as Pregl micro-pipettes) holding from 0·05–0·1 ml., which are washed out into the flask with a fine stream of water or acid.

After weighing out the substance (3–5 mg. of solid or up to 0·1 ml. of a liquid such as blood), all the material is washed down into the bulb of the flask with 1 ml. of pure concentrated sulphuric acid. To this is added about 150 mg. of the catalyst mixture, previously prepared by grinding together 32 parts by weight of potassium sulphate, 5 parts of mercuric sulphate, and 1 part of selenium powder.

The Kjeldahl flask is heated over a small flame so that the liquid boils slowly. After about 5 minutes the heating is increased so that the mixture boils vigorously, and boiling is continued for a further 15 minutes or until the solution becomes clear and straw-yellow in colour.* If the decomposition is incomplete in this time, the mixture, after cooling, should be treated with 2–3 drops of perhydrol and again boiled for 5 minutes.

2. Pre-reduction Processes for the Analysis of Nitro-compounds, etc.

(a) The complete reduction of many involatile compounds (e.g. azo dyes or nitro-amines) can often be effected by adding 100 mg. of glucose to the acid digestion mixture and then carrying out the initial heating very gently.

(b) Volatile compounds should be pre-treated in a bomb-tube as follows. The substance is weighed out into a bomb-tube (p. 91) treated with 1 ml. of hydriodic acid (sp. gr. 1·7), sealed up, and heated in a tube-furnace for 1 hour at 200° C.; or, for heterocyclic compounds such as quinoline, for 1 hour at 300° C. After cooling, the hydriodic acid is removed from the tip of the bomb-tube by warming it gently, after which the tube is opened in the usual way. The contents of the tube are then washed quantitatively into a micro-Kjeldahl flask, treated with 2 ml. of concentrated sulphuric acid, and decomposed as described in (c) below.

(c) Substances which do not volatilise can be weighed directly into the Kjeldahl flask and treated with 1 ml. of hydriodic acid to which has been

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* Decomposition is not necessarily complete as soon as the liquid becomes clear yellow, but 15 minutes' boiling is usually sufficient. Pyridine ring compounds, however, may require a much longer digestion time (2–4 hours). Cf. Shirley, R. L., and Becker, W. W., *Ind. Eng. Chem.* (Anal. Edn.), 1945, **17**, 437.

added a few grains of red phosphorus. The mixture is boiled gently for
$\frac{1}{2}$ hour. It is then cooled, the neck of the flask is washed down with water
until the bulb is half-full, and 2 ml. of concentrated sulphuric acid are added.

The diluted mixture is then heated, with shaking, so that the contents
boil vigorously, and the water and hydriodic acid distil off. When all the
hydriodic acid has been removed and the neck of the flask is free from
sublimed iodine, 150 mg. of catalyst (sulphate-selenium mixture) are added
and the whole is boiled up for a further 15–20 minutes. After cooling,
3 ml. of water are added and the ammonia distillation can be proceeded with.

(d) Diazo-compounds may be analysed by dissolving in three to four
times their weight of phenol, heating for a short time on the water-bath,
and then proceeding as in (a) or (c) above.
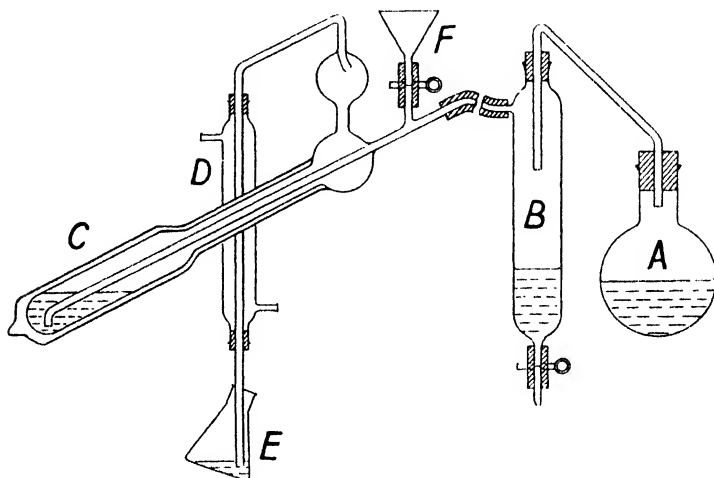
Fig. II.12.   Ammonia Distillation Apparatus of Parnas and Wagner.

3. *Ammonia Distillation*

(a) *Apparatus*. For the micro-distillation of ammonia a number of
different types of apparatus have been described. Fig. II.12 illustrates
that due to Parnas and Wagner.* It consists of a steam generator A,
followed by a water trap B, to which is connected a vacuum-jacketted
distillation flask C. This, in turn, is connected to a condenser D with a
foam-trap interpolated, and the condenser tube dips into the receiver E.
The distillation flask has a funnel F attached, through which alkali may be
introduced.

The condenser tube should be made either of clear quartz or of silver so
as to obviate errors due to solution of alkali from the glass.

The whole apparatus should be steamed out thoroughly before use.

* Parnas and Wagner, *Biochem. Zeit.*, 1921, **125**, 253.

(b) *Procedure.* The apparatus is washed in chromic acid and then distilled water, connected up, and steam is passed through for 10 minutes without cooling the condenser tube. The outside of the condenser tube is rinsed with distilled water and then the distillation flask is emptied from condensed water.

A steamed-out receiver (e.g. a 100 ml. Erlenmeyer flask) containing up to 5 ml. of $N/100$ hydrochloric acid and a few drops of screened indicator* is placed in position and the diluted contents of the Kjeldahl flask are introduced quantitatively into the distillation flask via funnel F. Four successive rinsings of the Kjeldahl flask with about 5 ml. of distilled water is adequate for this purpose. 15 ml. of 30% caustic soda are then added via the funnel and the tap is closed.†

The receiver is then raised so that the condenser tube dips below the surface of the liquid and then steam is allowed to pass through the apparatus for 5 minutes. The receiver is then lowered away from the condenser tube and the distillation is continued for a further 2 minutes, at the end of which the liquid on tube D is rinsed into the receiver E, which is then ready for titration.

The contents of the distillation flask C are emptied by removing the heat from the steam flask A, and so allowing a suck back of liquid into the water trap B to occur. After rinsing the distillation flask with distilled water in the same manner, the apparatus is ready for further use, and the water trap can be drained whenever convenient.

Alternatively to the Parnas and Wagner procedure given above, the method of Bang, described on p. 197, or the apparatus of Hoskins‡ may be used.

For estimating minute traces of ammonia, the Conway technique (described on pp. 205–207) is most suitable. Colorimetric methods (pp. 297, 304) can also be used.

(c) *Titration.* The contents of the receiver are boiled for a few minutes to remove carbon dioxide, cooled, and then titrated from a 5 ml. micros burette (graduated to 0·01 ml.) in which is contained $N/100$ caustic soda- or baryta. The end-point is reached when the neutral tint of the indicator, persists for 2 minutes.

In place of $N/100$ hydrochloric acid, 5 ml. of a cold saturated solution of boric acid may be used as absorbent for the ammonia. After carrying out the ammonia distillation this solution may be titrated directly, as an alkali, with $N/100$ hydrochloric acid, using as indicator screened methyl red/methylene blue. This modification of the analysis, due to Meeker and Wagner,§ obviates the need for storing carbonate-free $N/100$ alkali.

* Methyl red/methylene blue is advised: see p. 86.
† To destroy any mercury-ammonia complexes, the alkali should contain 5% of sodium thiosulphate.
‡ Hoskins, J. L., *Analyst*, 1944, **69**, 271.
§ Meeker and Wagner, *Ind. Eng. Chem.* (Anal. Edn.), 1935, **5**, 396.

THE DETERMINATION OF SULPHUR
AND OF THE HALOGENS

Principles

Several different micro-chemical methods have been proposed for the determinations of sulphur and of the halogens in organic compounds.

The classical **Carius method** (p. 90) can in the hands of an experienced analyst be relied upon to give, on the milligram scale, results of high accuracy provided that satisfactory hard-glass tubing is available. Careful practice in the techniques of micro-filtration (Part 1, pp. 22–25) is needed, however, together with rigorous attention to such factors as the assurance of complete oxidation and freedom from production of glass splinters when opening the bomb-tube. In common with other micro-gravimetric procedures, the percentage error of the estimation rises unduly ($>5\%$) if one attempts to collect and weigh less than 2 mg. of precipitate. This corresponds to 0·27 mg. of sulphur (as $BaSO_4$), 0·5 mg. of chlorine (as AgCl), or 1 mg. of iodine (as AgI). Though one should not attempt to oxidise more than 20 mg. of an organic substance in a micro-bomb-tube (see pp. 90–91), wet oxidation methods can be carried out both in sealed and open tubes with quite large quantities of material (up to 100 mg., see pp. 100–102) so that the micro-gravimetric procedures are to be recommended for the determination of traces (0·1–1%) of these and other elements in organic matter. When the required element comprises a very small percentage of the substance being analysed, special care must be taken to avoid contamination of the final precipitate by the occlusion of other material. Barium sulphate precipitates are particularly prone to this source of error.

The peroxide fusion method of Piria and Schiff, which on the macro-chemical scale has, by the use of the Parr bomb, become a safe and rapid method of sulphur estimation, is quite unsuitable for accurate micro-chemical work, since, owing to the amount of sodium peroxide used for the fusion and to the large volume of solution involved, the danger of co-precipitation of impurities is unduly high. It is valuable, however, for the estimation of *fluorine* (p. 191).

Robertson's rapid method* for estimating chlorine or bromine in organic compounds by decomposition with chromic and sulphuric acids and collecting the halides (in the form of free elements, halogen acids, or chromyl chloride) in alkaline hydrogen peroxide has been suggested for micro-chemical work by Zacherl and Krainick.† It needs most careful manipulation if losses of halogens are to be avoided and cannot be recommended for work of high accuracy.

* Robertson, P. W., *J. Chem. Soc.*, 1915, **107**, 902.
† Zacherl and Krainick, *Mikrochem.*, 1932, **11**, 61.

For the ultimate analysis of compounds containing not less than 5% of sulphur or of a halogen, the **combustion methods** introduced by Pregl have proved to be most reliable procedures for milligram-scale work, and when a final titrimetric procedure rather than a gravimetric procedure is adopted, they are also the most rapid analytical processes. The compilers recommend these combustion analyses most strongly for routine work, but advise that the improved techniques which are set out in pp. 84–90 should be followed in detail.

Combustion analyses, both for sulphur and for halogens, are carried out with a large excess of oxygen in a hot quartz tube containing contact strips of platinum which serve to catalyse the oxidation of organic matter. *Organic sulphur compounds* yield a mixture of SO_2, SO_3, and H_2SO_4, all of which can be collected in neutral hydrogen peroxide (which rapidly oxidises the sulphite) and subsequently estimated as sulphate. Precautions have to be taken both to prevent the deposition of sulphur trioxide inside the combustion tube and also to prevent the formation of an uncondensable sulphuric acid mist. Both these contingencies are obviated by carrying out the combustion in moist oxygen (pp. 86–87).

Chlorine and *bromine* compounds yield a mixture of the free halogens and of their hydrides. These, too, can be collected quantitatively in dilute hydrogen peroxide, which easily reduces free halogens. For the analysis of compounds containing a large percentage of halogen it is preferable, however, to use alkaline sodium bisulphite as the absorbent. This retains free halogens rather more effectively in the cold, though the excess of sulphite must subsequently be destroyed by oxidation with hydrogen peroxide.

Combustion analyses for *iodine* are apt to give trouble owing to the separation of the solid element in the cool portion of the combustion tube. Care must be taken that the absorbent, which in this case *must* be alkaline bisulphite, is sucked well up into the combustion tube at the end of the analysis. Moreover, on account of the high molecular weight of iodine it is not advisable to carry out this estimation with less than 10 mg. of substance if accurate results are required. Even then, to enhance the sensitivity of the final titration, one should follow the procedure of Leipert,[*] whereby the iodine is oxidised with bromine in acetic acid to iodic acid, which, after the removal of the excess of bromine with formic acid, is treated with potassium iodide solution, whereupon six times the original quantity of iodine is liberated.

$$I_2 + 5Br_2 + 6H_2O = 2HIO_3 + 10HBr$$
$$HIO_3 + 5HI = 3I_2 + 3H_2O$$
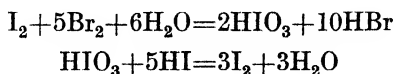
The sensitivity of these combustion methods of analysis depends essentially upon the titrimetric procedure that is chosen. These are

* Leipert, *Mikrochem.*, Pregl Festschrift, 1929, 266.

discussed on pp. 87–90 and 180–184, but it may be noted that the lower limit for determination of sulphur or chlorine is about 3 μg. and for iodine about 2 μg. Combustion of 5–10 mg. of an organic substance should, however, give results accurate to 0·1%.

Zimmermann's method* of estimating sulphur by reducing the substance in a sealed tube with hot potassium, and subsequently decomposing the resultant potassium sulphide with dilute acid in a hydrogen atmosphere so as to give hydrogen sulphide which is then absorbed quantitatively in cadmium acetate solution and finally estimated iodimetrically, is of recent origin. The complete estimation takes about 30 minutes.

Though the technique is elaborate it is reputed to give accurate results with sulphur compounds of all types, and can be recommended for trial by an experienced micro-analyst.

A. The Combustion Method of Analysis for Sulphur and for Halogens

The general theory of this analysis has been indicated in the preceding pages. The same combustion technique can be used both for sulphur and for the halogen elements, though special care must be taken to avoid losses when dealing with iodine compounds (see p. 89). It is possible (i) to determine both sulphur and a halogen in the same sample of material, and (ii) to determine iodine in the presence of the other halogen elements. The estimation of *fluorine*, of course, requires an entirely different technique (p. 191).

APPARATUS† (see fig. II.13)

Oxygen from a cylinder is metered through a Pregl pressure regulator and then freed from traces of organic vapours or sulphur compounds by passage through an electrically heated tube of hard glass *A*, maintained at about 350° C. and filled with a 1 : 1 mixture of cerium dioxide and lead chromate supported on pumice granules of 10–14 mesh. The purified gas is then passed through tap *B* and the water bubbler *C*, so as to saturate with water vapour (but not to deposit liquid in) the oxygen, which then enters the combustion tube via the side-arm. If necessary the bubbler *C* is warmed slightly.

The organic substance is burned inside the combustion tube *D–G*, which should be constructed of transparent quartz tubing. The horizontal part of the tube *DE* holds a quartz rod baffle 8 cm. long, and then the boat containing the substance being analysed. The narrowed vertical section *EF* holds the platinum contacts (see below), whilst the lower portion is filled with small glass beads and ends at an A10 cone-joint. Naturally, the sections *EF* and *FG* of the combustion tube must be sealed together after

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* Zimmermann, W., *Mikrochem.*, 1943, **31**, 15; 1947, **33**, 122.
† Cf. Ingram, G., *Analyst*, 1944, **69**, 267.

they have been filled.   The inlet tube J, of which the outer end is normally closed by a rubber stopper, serves to wash down section FG on completion of the combustion.

The vertical section EF of the quartz tube, together with the final 5 cm. of the horizontal section DE, are built into an electric heater (made by winding nichrome wire on a layer of wet asbestos paper, then lagging externally with layers of wet asbestos, and firmly binding together with asbestos string).   The furnace winding should be brought down as closely as possible to the quartz side-tube J.   This heating furnace should be set, by a rheostat, to give a steady temperature of not less than 800° C.
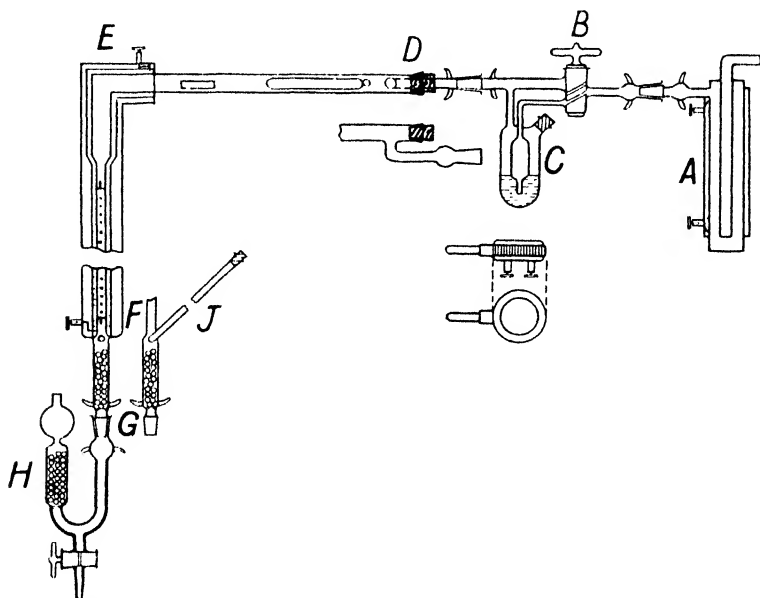
Fig. II.13.   Combustion Apparatus for Estimation of Halogens or of Sulphur.

The *platinum contacts*, with which the quartz tube is packed between E and F, should be made from 0·05 mm. thick platinum foil.   A strip 12 cm. long and 17 mm. wide is bent longitudinally against a piece of stiff cardboard so that its cross-section forms a Latin Z and fits snugly into EF. Before the contact is inserted into the combustion tube it should be cleaned by boiling with dilute nitric acid and then igniting strongly.   If for any reason it becomes poisoned (e.g. by arsenic or other volatile metal) it should be etched with hot aqua regia.

Care should be taken to ensure that the inside of the quartz combustion tube is not contaminated by dust when the apparatus is not in use.

Detachable absorbers can be made to various designs.   One convenient pattern, H, consists of a Pyrex-glass **U**-tube with a tap at the bottom and an open limb, packed with glass beads, terminating with a small splash-bulb.

For the estimation of iodine, a narrow vertical tube, dipping into a small flask containing the absorbent solution, is preferable.

The ground-glass connections at D and GH should be moistened with distilled water, not grease, and secured by springs.

Absorption Reagents

1. *Neutral hydrogen peroxide solution* should be used for the determination of *sulphur*. It can also be used for the collection of *chlorine* or *bromine*.

The reagent should be prepared freshly for each analysis by adding 5 drops (approximately 0·3 ml.) of 10-volume halogen-free hydrogen peroxide to 10 ml. of distilled water and then neutralising the solution carefully with $N/100$ sodium hydroxide, using as indicator 2 drops of a 0·05% screened methyl-red/methylene-blue mixture (compare p. 134).* It is advisable to check the purity of each stock sample of hydrogen peroxide.

2. *Alkaline sodium bisulphite solution* is the best absorbent to use for the determination of the *halogens*. *It* must *be used for the determination of iodine*, and is preferable to hydrogen peroxide for the retention of fairly large (over 2 mg.) quantities of chlorine or bromine.

The reagent is prepared by adding to 10 ml. of distilled water 5 drops each of saturated solutions of halogen-free sodium bisulphite and of sodium carbonate.

When this reagent is used for the absorption of chlorine or bromine, the reaction product must be boiled for 2 minutes with hydrogen peroxide (about 3 drops of 100-volume reagent) to destroy the excess of sulphite before proceeding to either the gravimetric precipitation or the titrimetric estimation (p. 87).

For the determination of iodine, the oxidation is completed by use of bromine in acetic acid/sodium acetate solution (p. 89).

General Combustion Procedure

After cleaning and drying, the apparatus is assembled with the bubbler C filled to the neck with distilled water, and the temperatures of the main furnace and of the oxygen purifier are brought to the values already given (pp. 84–85), whilst a stream of moist oxygen, at the measured rate of 20 ml. per minute, is passed through the combustion tube. Iodine combustions require a slower flow rate of 6 ml. per minute.

After inserting the weighed sample in its boat, and at its rear a quartz baffle, the combustion of the substance is carried out with a gas flame. As usual, the tube DE is first heated at the rear end, beneath the baffle, and then the flame is moved forward gradually until the boat is directly heated and the substance burns away completely in the oxygen stream.

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* It is convenient to keep in separate dropping bottles separate solutions of (*a*) 0·05% methyl red and (*b*) 0·05% methylene blue in alcohol. For each titration use 4 drops of methyl red and 2 drops of methylene blue solution to prepare the screened indicator.

This operation should take not less than 10 minutes; after a further 10 minutes the oxygen stream may be turned off, the absorber detached, and the cool portion FG of the combustion tube washed out with a stream of water from the jet J. The absorber and the washings, totalling about 10 ml., are then collected for analysis, whilst if necessary a further combustion can be commenced as soon as DE is cool.

Quantitative Estimation of Combustion Products

A. Gravimetric Procedures

The standard procedures, due to Pregl, for the determination of sulphur as barium sulphate and of chlorine and bromine as their silver salts are given below. These operations are very accurate, but time-consuming.

1. For the determination of *sulphur*, the absorbent (H_2O_2) and washings are collected in a platinum or steamed-out resistance glass dish, treated with 1 ml. of a filtered 10% solution of barium chloride and 10 drops of N hydrochloric acid, and evaporated on the water-bath, under a dust-cover, to dryness. The residue is washed with 2 ml. of 25% hydrochloric acid and again evaporated to dryness. By this procedure there is produced a coarse, granular precipitate which can be filtered easily.

The residue is then treated with a few millilitres of distilled water, filtered through a micro-crucible, washed with 1% hydrochloric acid, and dried at a dull red heat (p. 24).

2. For the determinations of *chlorine* or *bromine*, the absorbent and washings are collected in a small test-tube, acidified with 1 ml. of concentrated nitric acid, and then treated with 2 ml. of a 5% solution of silver nitrate. After heating on the water-bath for 15 minutes the precipitate aggregates, and may then be filtered through a Pregl micro-filter (see fig. I.9, p. 23), washed with 1% nitric acid, then alcohol, and dried at 110° C. in a Pregl drying block (p. 29).

B. Volumetric Procedures

1. Estimations of Chlorine or Bromine

The foregoing method of combustion analysis converts organic chlorine or bromine to halide anions, whichever absorbent is used, and provided that any excess of sulphite or of hydrogen peroxide has been removed completely, any standard method of halide titration may be used. (Compare pp. 180–184 of Part III.)

However, the volumetric procedure of Vieboch,* involving the use of *mercuric oxycyanide*, is particularly convenient and accurate for this analysis. Mercuric oxycyanide is water-soluble, neutral, and unionised. It reacts

* Vieboch, *Ber.*, 1932, **65**, 496.

quantitatively with neutral halides, liberating an equivalent of alkali, which may be titrated acidimetrically, e.g.

$$Hg(OH)CN + NaCl = HgClCN + NaOH$$
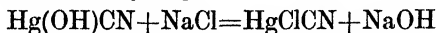
The reagent solution is prepared by shaking 20 g. of solid mercuric oxycyanide (this can be purchased easily) with 1 l. of distilled water and filtering the stock solution (which is slightly alkaline) into a brown glass bottle.

For each estimation neutralise 10 ml. of this solution with $N/100$ sulphuric acid, using as indicator screened methyl red/methylene blue, and add this to the exactly neutralised solution of the halide to be estimated. Titrate the mixture to neutrality with $N/100$ sulphuric acid.

Accurate results from combustion analysis will only be obtained if the halide solution is boiled for about 10 seconds to expel carbon dioxide before the initial neutralisation.

2. Estimation of Sulphur

(a) The combustion product from a substance containing only C, H, O, S is merely dilute sulphuric acid containing a little carbon dioxide. The absorbent solution may therefore be titrated directly with dilute ($N/100$) alkali, after removing the carbon dioxide by boiling for about 10 seconds. Screened methyl red should be used as the indicator.

(b) If elements such as nitrogen or halogens have also been present, then the combustion products contain other acids (HNO_3; HCl). In the *absence of nitrogen*, the *halogens* and *sulphur* may be determined independently by alkali titration as follows*:

(i) Collect the absorbent solution (H_2O_2) and washings, boil to remove carbon dioxide, cool, add screened indicator, and titrate to neutrality with $N/100$ caustic soda. This gives $H_2SO_4 + HCl(HBr)$.

(ii) Then add 10 ml. of neutral mercuric oxycyanide reagent and again titrate the liberated alkali with $N/100$ sulphuric acid. This gives HCl(HBr) alone.

(c) If nitrogen is present, then the formation of some free nitric acid complicates the titrimetric procedure for sulphur. By precipitating an equivalent of barium sulphate from *a known weight* of barium chloride, evaporating, without filtering, and so removing all free acid, there is left only a mixture of $BaSO_4$ and $BaCl_2$ in which the *excess* of barium chloride can then be determined by means of mercuric oxycyanide.† The exact procedure is as follows:

Collect the absorbent solution and washings in a clean dish and evaporate on the water-bath until almost dry. Add 1 ml. of 0·1N hydrochloric acid and a weighed quantity (20–50 mg.) of pure crystalline barium chloride ($BaCl_2$, $2H_2O$) and again evaporate. Then add 4 ml. of distilled water and

* Gibson and Caulfield, *Analyst*, 1935, **60**, 522.
† Ingram, *Analyst*, 1944, **69**, 267.

evaporate until all the free acid has been removed. Wash the resultant mixture of barium sulphate and barium chloride into a 150 ml. conical flask, add 10 ml. of neutralised mercuric oxycyanide reagent, and screened indicator, and titrate to neutrality with $N/100$ sulphuric acid.

The volumetric methods described above can also be used in conjunction with the Carius method of decomposition of organic compounds. By the use of micro-burettes results accurate to 0·1% can easily be obtained from the combustion of ca. 5 mg. of an organic substance.

Procedure for Estimation of Iodine by Combustion

When using the apparatus of fig. 11.13 for the determination of iodine, fit to the end G of the combustion tube, by a ground joint, a piece of thick-walled tubing, about 14 cm. long, dipping into a 150 ml. conical flask and proceed as follows:

(i) Prepare a little fresh bisulphite mixture (p. 86) and pour 2 ml. of this solution through the side-tube J of the combustion apparatus, so that the beads are thoroughly wetted and the excess of liquid drains into the conical flask, and add to the flask 5 ml. of a 10% sodium acetate solution.

(ii) Carry out the usual combustion *slowly*, using not less than 7 mg. of substance, and adjusting the oxygen flow rate so that only 1 bubble per second passes out through the sodium acetate. An explosion of the organic vapour vitiates the whole analysis.

When the movable burner has been advanced so that all the carbon has been burnt away, continue the heating until all the iodine has been driven over into the visible portion of the tube FG, forming a brown ring of dissolved iodine about one-third of the way down the bead column with a clear zone above.

(iii) To wash out the apparatus add 3–4 drops of bromine to 5 ml. of a 10% solution of sodium acetate in 95% acetic acid contained in a 10 ml. micro-beaker. Add 2 ml. of this mixture to the bead tube through the side-tube J and rinse down with a few millilitres of water from a wash-bottle. Disconnect the thick-walled tube and rinse it, both inside and out, into the conical flask with water. Then place the micro-beaker containing the bromine solution under the bead tube FG and apply suction at J until the beads have been covered thoroughly with solution. Finally rinse out the micro-beaker into the conical flask, and then rinse out the bead tube, adding these washings too.

If the resultant solution is colourless, owing to the complete reduction of the bromine by excess of bisulphite, add bromine-water until a slight excess is present. This ensures the complete oxidation of the iodine to iodic acid.

(iv) Before titrating for iodic acid remove the excess of bromine by adding a few drops of pure formic acid, testing the solution with a drop of methyl red indicator, which will be discoloured so long as free bromine is present.

Then to the bromine-free solution add 2 ml. of a 10% solution of potassium iodide and 5 ml. of $2N$ sulphuric acid. Stopper the flask and leave it for 5 minutes to complete the reaction:

$$HIO_3 + 5HI = 3I_2 + 3H_2O$$
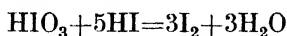
Wash down the stopper and then titrate with $N/100$ sodium thiosulphate. This reagent should be run in rapidly until the solution becomes pale yellow, 4 drops of starch solution should then be added and the titration then continued carefully until the blue colour vanishes.

By this procedure 1 ml. of $N/100$ thiosulphate is equivalent to 0·2115 mg. of iodine.

NOTE.—The presence of chlorine or of bromine does not interfere with the determination of iodine by the above method.

If compounds contain chlorine or bromine in addition to iodine it is possible to carry out the combustion for halogens as described above, collecting the products in bisulphite mixture and rinsing this through the conical flask with more bisulphite solution and then water. By adding hydrogen peroxide solution and boiling, all the iodine may be removed, leaving intact the other halogens, which may then be estimated by the procedure given on p. 87.

B. Micro-Carius Procedure

Elements other than C, H, O, N can be estimated in any organic compound by decomposing the material with an excess of fuming nitric acid at *ca.* 300° C. inside a sealed bomb-tube, and, after opening the tube when cold and washing out the contents, estimating the inorganic residue by standard gravimetric methods. This standard procedure is equally accurate on the milligram scale, provided that bomb-tubing of high quality is obtainable. It is advisable to test each new batch of tubing to see (*a*) that it does not splinter after heating under pressure and (*b*) that it is not etched by the hot reagents. Many samples of resistance glass have a tendency to flake at the acid surface, giving traces of silica powder inseparable from the precipitate which is to be weighed.

For work on the milligram scale bomb-tubes of 2 mm. wall, 1 cm. external diameter, and about 20 cm. length should be used. Larger bomb-tubes and correspondingly larger quantities of material must of course be used for the determination of "trace elements."

Many convenient patterns of bomb-tube furnaces giving regulated temperatures up to 350° C. can now be purchased, though a suitable electrically heated furnace can be made quite simply by any competent mechanic. The furnace should be mounted against a substantial brick wall and behind a metal shield, so that there can be no danger from flying glass in the event of an explosion.

Decomposition of the Organic Substance

For the analysis of 3–10 mg. of substance, the clean, dry bomb-tube should be charged with 0·5 ml. of pure *fuming* nitric acid (density 1·5), which should be added through a long funnel so that the sides of the bomb-tube are not wetted. If *halogens* are to be estimated, about three small crystals of solid silver nitrate should be added also.

The substance for analysis, if solid, should be weighed out in a capillary tube about 3 cm. long and 1–2 mm. bore, open at both ends. For analysis of liquids, fine capillaries about 5 cm. long and 0·5–1 mm. bore should be used (compare p. 67).

Owing to the large excess of acid which is used, the decomposition of nearly all substances can be completed by heating to 300° C. for 6 hours*: this is most conveniently carried out over-night. The tube should be both heated up and cooled down gradually, and must not be opened until it is *quite* cold.

The sealing and the opening of micro-bomb-tubes is carried out by the standard procedure for macro-chemical work.† The sealing of resistance glass must be carried out with an oxy-gas flame, and the end of the tube must be drawn out evenly into a tapering capillary without decreasing appreciably the thickness of the glass at any point. After the heating process the pressure in the tube is released by inserting the tip of the capillary seal in a gas flame and allowing the gaseous products to release themselves by blowing out through the softened glass. The tube is then removed from the furnace, wiped externally. scratched around the base of the capillary seal with a fine glass-knife, and opened by touching the cleaned scratch with the point of a heated glass rod. The top of the tube is removed by a sharp tap and, after inspection, the sharp edges of the open end of the tube are rounded off in a flame. It is necessary to take extreme precautions against the formation of glass splinters, and immediately a tube has been opened it is essential to inspect it carefully with a lens to ensure its cleanliness and freedom from adhering glass particles.

SUBSEQUENT GRAVIMETRIC PROCEDURES

1. Halogens. For halogen analysis, silver nitrate is added to the initial contents of the bomb-tube. Hence dilute the mixture inside the tube with distilled water, extract the small capillary with a platinum wire sealed into the end of a glass rod, rinse it clean, warm-up the diluted solution on a water-bath, and finally filter off the precipitated silver halide through a Pregl micro-filter merely by inserting into the bomb-tube the suction

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* Longer heating may be required for the complete oxidation of: sulphoxides and sulphones, highly chlorinated aromatic compounds, some quinonoid dyes, and organometallic compounds such as phthalocyanines.

† Cf. Gattermann-Wieland, "Practical Methods of Organic Chemistry," pp. 70–71 (English translation, 1938).

capillary attached to the filter-tube (fig. I.9, p. 23). The precipitate, after washing with 1% nitric acid and then alcohol, should be dried at 110° C. and weighed.

If the presence of traces of glass is suspected, a precipitate of silver chloride can be dissolved out of the filter-tube with warm dilute ammonia and reprecipitated with dilute nitric acid. Also, all silver halides can, after weighing, be dissolved out by means of potassium cyanide solution, and after thorough washing the filter-tube together with any glass can then be redried and reweighed.

2. Suphur. The contents of the tube are washed out into a small dish of platinum or resistance glass, filtered if necessary, and evaporated almost to dryness on the water-bath. Precipitation with barium chloride is then carried out as described on pp. 87–88.

3. Selenium.* The contents of the tube are washed out into a boiling tube with water and concentrated hydrochloric acid and heated on the water-bath. A slow stream of sulphur dioxide is then passed in. This destroys the nitric acid and also precipitates the selenium, which eventually settles as a black powder, though initially it may separate as a reddish colloid. After heating for 20 minutes, the sulphur dioxide lead is removed and rinsed, the liquid is allowed to cool, and the elementary selenium is collected in a Pregl filter-tube, washed with water and alcohol, dried at 110° C., and weighed.

4. Tellurium.* The contents of the tube are washed out into a dish and the nitric acid is removed by evaporation. A little water is added and the tellurium is precipitated as the free element by adding 3 ml. of saturated aqueous sulphurous acid and 2 ml. of a 15% aqueous solution of hydrazine hydrochloride. The mixture is heated on the water-bath for 10 minutes, and, after the addition of a further 2 ml. of sulphurous acid solution, the precipitate is sucked over into a filter-tube, washed with hot water, then alcohol, dried at 110° C., and weighed.

5. Arsenic. The contents of the tube are washed into a dish and evaporated to dryness on the water-bath. The residue is taken up in 5 ml. of $2N$ ammonia solution and the arsenic is precipitated with 1 ml. of magnesia reagent, prepared by dissolving 5·5 g. of magnesium chloride and 10·5 g. of ammonium chloride in 100 ml. of distilled water. After standing overnight, the magnesium ammonium arsenate is filtered into a Neubauer crucible, as described on p. 25, ignited, and weighed as magnesium pyroarsenate. More rapid methods for the determination of arsenic are given on pp. 173–175 and 278–280.

* Drew and Porter, *J. Chem. Soc.*, 1929, 2091.

6. Phosphorus. Phosphorus may be determined gravimetrically as magnesium pyrophosphate, following the procedure described above for arsenic.   A more rapid volumetric method of estimation is given on p. 164.

7. Mercury. Mercury may be precipitated as sulphide by means of hydrogen sulphide and collected as such, but it is more accurate to redissolve the precipitate in chlorine-water and then estimate the element by electro-deposition (p. 494).   Analysis by direct combustion (p. 98) involves fewer manipulative errors.

THE DETERMINATION OF OXYGEN

Principles. The percentage of oxygen in an organic substance is usually arrived at by difference. It thus is a figure which is subject to the accumulation of errors; these may become very serious if the material contains some inorganic contaminant (e.g. traces of glass abraded from a filter-plate) or if any of the combustions have not been effected completely. Consequently reliable absolute methods for the determination of oxygen have long been sought.

Considerable progress has been made along lines indicated by Ter Meulen* who hydrogenated organic substances catalytically and so converted the oxygen to water, but it now seems that the better procedure is one which leads to the quantitative separation of oxygen in the form of *carbon monoxide*. This second method, which has been elaborated for micro-analysis chiefly by Schütze,† involves the pyrolysis of the organic compound in an atmosphere of purified nitrogen to give hydrocarbons, carbon oxides, and water which are then passed over a column of graphite heated to 1,150° C. At this temperature the quantitative conversion of both steam and carbon dioxide to carbon monoxide can be effected.

The exit gases are then passed over pellets of caustic potash to remove acidic vapours which may arise from nitrogen, sulphur or halogen compounds, and then through warm iodine pentoxide, with which the carbon monoxide reacts in accordance with the equation

$$I_2O_5 + 5CO = I_2 + 5CO_2$$
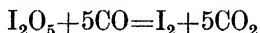
The iodine sublimes off and is trapped in a potash tube, from which it is finally washed out and estimated volumetrically in the usual manner (p. 89). Alternatively the carbon dioxide may be collected and weighed.

Though this method appears to be capable of yielding results of 0·2–0·5% accuracy, it needs great care, and it is advisable to minimise errors by conducting blank analyses. It can only be recommended when long series of analyses are called for and the time necessary to build and test the special apparatus which is needed can be justified. The essential details of this method are outlined below.

Apparatus. An electrically heated, high-temperature, combustion apparatus of standard design (fig. II.2) is used together with a quartz tube (fig. II.4) containing as its main packing a 12 cm. column of powdered graphite or pelletted carbon black held in position with quartz chippings. The outflowing gases from the beak end of the tube are passed firstly through

* *Rec. Trav. Chim.*, 1922, **41**, 509.

† *Z. Anal. Chem.*, 1939, **118**, 241; see also Zimmermann, idem., p. 258; Unterzaucher, *Ber.*, 1940, **73**, 391; Aluise, Hall, Staats, and Becker, *Analytical Chemistry*, 1947, **19**, 347.

a short tube containing pellets of caustic potash and then through a straight tube, 12 cm. long and 1 cm. diameter, which is packed with pure iodine pentoxide maintained at 120° C. by a surrounding heating mortar (p. 63) containing boiling glacial acetic acid and fitted with a water condenser. The exit gases containing the iodine vapour are then passed into a bead or spiral-filled absorber moistened with 20% caustic potash solution.

Nitrogen gas from a cylinder is purified by passing through a pre-heater (see p. 64 and fig. II.3) containing reduced copper at 250–400° C. and then through soda-lime pellets and anhydrone. A by-pass tube, inserted by means of a **T**-joint and tap between the combustion tube and the absorption train, allows even the filling end (*X* of fig. II.4) of the quartz tube to be swept out with nitrogen during the introduction of the sample and before the substance is heated, though subsequently the pure nitrogen is introduced in the normal way through the side-arm (*K* of fig. II.4). It is advisable to close *X* with a short tube which terminates in a capillary tap.

General Procedure.* With the exception that nitrogen is used instead of oxygen, the substance is decomposed in a platinum boat according to the standard combustion procedure described on p. 68. Great care is taken to remove all traces of oxygen by sweeping out the filled tube for 10 minutes in each direction before connecting the absorbers and switching on the movable electrical heater (*B* of fig. II.2) which surrounds the substance. The temperature of the main heating furnace (*A* of fig. II.2) is maintained throughout at 1,150° C., whilst the temperature of furnace *B* is gradually increased up to 1,000° C. during 40–50 minutes. Throughout this time the pure nitrogen is passed through the tube at a steady rate of 8–10 ml. per minute, which may be checked by the use of either a flowmeter or a Mariotte bottle. After maintaining the full temperatures in both furnaces for 15 minutes the absorption-tube which has collected the iodine is detached, washed out with water into a beaker containing bromine in sodium acetate solution, and thereby the iodine is all converted to iodate. Subsequently this iodine is estimated volumetrically by Leipert's method, exactly as described on p. 89.

0·1333 mg. of oxygen is equivalent to 1 ml. of 0·02N thiosulphate.

Before commencing a series of estimations the carbon-packed tube should be heated for several hours in the stream of pure nitrogen. Even then it is essential to carry out a blank estimation using an empty boat and to note the amount of iodine which is then liberated. Only in this way is it possible to minimise the error due to the last traces of occluded oxygen or water vapour.

* The original literature should be consulted for full details.

THE DETERMINATION OF METALS IN ORGANIC SUBSTANCES

IT is rarely possible to estimate accurately a metal in an organic substance by weighing the residue left after carrying out the standard combustion analysis for carbon and hydrogen. Gold, silver, and the platinum metals can, however, be estimated in this way if the combustion has been carried out so carefully that there has been no sputtering of the contents of the combustion boat.

Other metals leave a residue often contaminated with carbonaceous material (compare p. 67), whilst some, such as mercury, arsenic, or lead, may in part volatilise, especially if the initial decomposition has been carried out in a reducing atmosphere.

In all cases, therefore, it is advisable to carry out a separate analysis for the metallic radical by destroying the organic matter under controlled conditions, and then determining the metal either gravimetrically as free metal, sulphate, or oxide, or by a volumetric or colorimetric process. The latter techniques, which are described in greater detail in Parts III and IV, are more particularly applicable to the estimation of "trace elements," but are the most convenient for the estimation of *arsenic* and of *antimony* in organic compounds.

Estimations of involatile metals when present in appreciable percentages (over 10% in organic substances) are most simply carried out gravimetrically, by first burning off the organic matter in an open boat or crucible. Arsenic, antimony, and mercury cannot be determined in this way. Compounds containing these elements, as also volatile organo-metallic compounds of zinc, cadmium, tin, lead, thallium, bismuth, etc., should be decomposed by wet oxidation methods, either in sealed Carius tubes (pp. 90–93) or in Kjeldahl flasks (pp. 100–102).

Determination of Metals as Such, or as Oxides

Metallic elements which can be determined as such, or as their oxides, are most simply estimated by oxidation inside platinum or porcelain microcrucibles. These should be about 10 mm. diameter, 12 mm. in height, and must be provided with lids.

Silver, gold, and the *platinum group of metals* are determined as such; *iron, aluminium,* and *chromium* as the oxides R_2O_3; *copper* and *magnesium* as the oxides RO; *silicon* and *tin* as the oxides RO_2. *Cobalt* and *nickel* are best determined by ashing the substance in a porcelain boat in an atmosphere of hydrogen and weighing the residue as the metal.*

* Friedrich, "Die Praxis der Quant. Mikroanalyse," p. 125.

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The following general procedure should be used. Clean the crucible with nitric acid, ignite it for 5 minutes, and then cool and weigh (p. 17). Add 2–5 mg. of substance and reweigh. Then place the crucible on a larger platinum lid supported on a quartz triangle or inside a larger porcelain crucible. Heat gently with the non-luminous flame of a Bunsen burner till the substance has charred, then increase the flame gradually and heat strongly for 5 minutes. Remove the burner and when the crucible is cool examine the residue for black specks of carbon. If any are present add a drop of dilute (1 : 1) nitric acid and reheat very carefully. Finally cool, weigh accurately, and then reheat and reweigh until the weight is found to be constant.

Alternatively the organic matter can be decomposed in a platinum boat inside a *Pregl micro-muffle* (fig. II.14). This is the best procedure for the
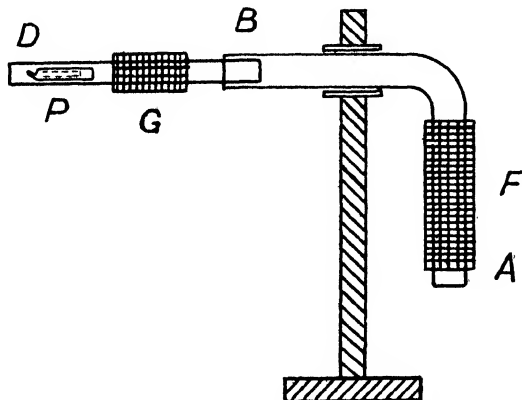

Fig. II.14. Pregl's Micro-muffle, for Estimation of Metallic Elements.

decomposition of organic salts of the alkali and alkaline earth metals, which are most conveniently estimated as sulphates.

The Pregl micro-muffle consists of a bent piece of quartz or Supremax glass tubing *AB* into the short horizontal limb of which is inserted a 20 cm. length of combustion tube *BD*, held firmly by wrapping the joint *B* with asbestos paper. The vertical limb *A* of the wider tube is wrapped with a double layer of wire gauze *F*, and is heated by the flame of an inclined Bunsen burner. This produces an ascending current of hot air which sweeps over the organic substance and carries away all volatile products.

The pyrolysis of the organic compound itself is carried out in a platinum boat, which, to prevent loss of material through sputtering, is surrounded by a platinum cylinder *P*, 3 cm. long and about 8 mm. diameter, made from foil and fitted at one end with a hook for facilitating its withdrawal from the combustion tube. To ensure even heating from a Bunsen flame, a roll of gauze *G*, about 5 cm. long, is placed loosely around the combustion tube over the platinum cylinder.

Determination of Metals as Sulphates

The procedure for the gravimetric determination of alkali and alkaline earth metals, including *sodium, potassium, calcium, barium,* and also *cadmium* and *manganese* is as follows:

Clean and ignite the platinum boat and cylinder; after cooling, weigh both articles together, then place 3–5 mg. of substance in the boat and reweigh. Transfer the boat to a copper block and add, from a fine capillary pipette, one drop of 20% sulphuric acid. Then place the boat inside the platinum cylinder, transfer both to the micro-muffle, and push in till the near end of the cylinder is 1 cm. from the open end *D* of the combustion tube. Light both burners, and at first heat the combustion tube about 3 cm. away from the platinum boat on the in-draught side. When combustion has commenced, gradually move the gauze roll *G* and the burner towards the substance. Eventually fumes of sulphur trioxide will appear at the mouth of the combustion tube.

When this fuming has ceased, move up the burner and gauze until the former is directly beneath the platinum boat. After a few more minutes remove the gauze roll *G* and heat the boat and cylinder directly in the full Bunsen flame for 10 minutes. Then allow to cool, transfer the platinum apparatus to a copper block, and eventually reweigh.

If the residue contains any black specks, indicative of incomplete combustion, hold the boat, with platinum-tipped forceps, in a flame for a few seconds, and then cool and reweigh.

NOTE 1. *Lead* compounds can cause trouble, owing to the ease of reduction to the free metal which could alloy with and destroy the platinum boat, and, in part, volatilise. Lead compounds should therefore be treated in a porcelain boat with a mixture of nitric and sulphuric acids, and a further drop of nitric acid should be added before heating the residue sufficiently strongly to drive off the excess mineral acids.

NOTE 2. *Lithium* compounds should be weighed out inside a stoppered weighing-bottle, as the resultant lithium sulphate is hygroscopic.

NOTE 3. Individual metals in mixtures (e.g. Na and K) can, of course, be estimated by dissolving up the weighed sulphates, and then determining the individual inorganic components by characteristic micro-chemical procedures.

Determination of Mercury by Combustion

APPARATUS (fig. II.15). A beaked micro-combustion tube *AB*, 40 cm. long, is packed to within 5 cm. of the beak with a 12 cm. column of small granules of quicklime, held between plugs of coarse asbestos, and is fitted inside a 15 cm. long cylindrical furnace so that only the packed section of the tube is heated. Over the beak of the combustion tube is placed a small tube of thin glass *CD*, 11 mm. in diameter and 5 cm. long, drawn out at one

end to a beak 3 mm. in diameter and 2 cm. long.   This tube is packed with
a 2 cm. layer of gold leaf or gold wire.

During the combustion a slow stream of air, from a gas holder, is dried
in a sulphuric acid bubble-counter E and a **U**-tube filled with anhydrone F
and passed into the combustion tube at the wide end.

A Bunsen burner, with a flame-spreader of wire gauze, is used to combust
the substance.

PROCEDURE. The combustion tube is ignited at *ca.* 700° C. in a stream of
dried air.   Meanwhile the gold-packed tube is warmed in a stream of dried
air, then cooled in a desiccator, wiped, and weighed accurately.

The open end of this tube D is then fitted over the beak B of the com-
bustion tube, and the Mariotte bottle M is attached to the other end.

The substance for analysis (3–10 mg.) is weighed out in a porcelain boat,
introduced into the combustion tube to within 5 cm. of the lime filling, the
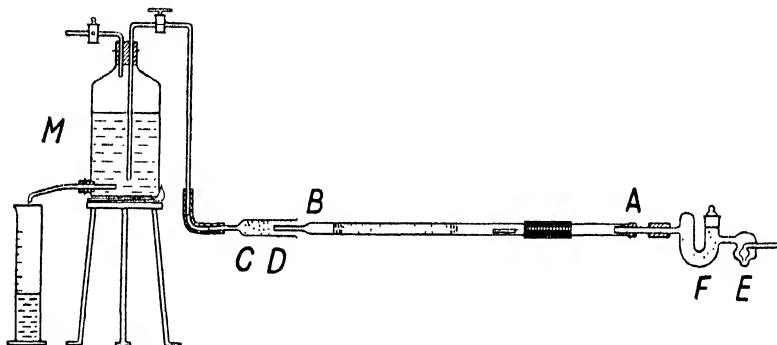

Fig. II.15.   Apparatus for Determination of Mercury by Combustion.

drier and bubble-counter are connected on, and air is passed through at the
measured rate of 6 ml. per minute.   The arm of the Mariotte bottle is then
lowered so that the outflow of water indicates that the air-speed through the
gold-packed tube is definitely greater than this.

The organic substance is then combusted as usual, by gradually moving
forward the Bunsen burner until the vapours have distilled into the calcium
oxide layer, where all sulphur or halogens are retained, and the mercury
has passed forward into the cool, empty part of the tube.

The gold-packed tube is then cooled by placing over it a piece of wet
chamois-leather, and the mercury is systematically driven over into it by
warming the front end of the combustion tube with a micro-burner.   After
aspirating through it about 1 l. more of air, the gold-packed tube is detached,
wiped externally, allowed to cool for half an hour inside a desiccator con-
taining calcium chloride, and reweighed.

WET OXIDATION METHODS

THE usual technique of burning off organic material in a flame or furnace and performing subsequent estimation for metallic substances on the ash is definitely contra-indicated when loss by volatilisation is a possibility. Resort is then made to methods of wet oxidation, of which estimation of nitrogen by the classical Kjeldahl procedure is typical. This and comparable processes are used in the estimations of trace elements in organic material (e.g. foods, blood, plants, fabrics, etc.). The elements most likely to be lost by muffling are the non-metals *phosphorus*, *chlorine*, and *sulphur*, and the more volatile elements such as the *alkali metals*, *arsenic*, *mercury*, *antimony*, *cadmium*, and *lead*. Naturally, the method of wet oxidation to be used depends upon the metal involved and on the subsequent analytical procedure. For instance, in the estimation of sulphur one cannot use sulphuric acid oxidation and in the estimation of chlorine the use of perchloric acid is contra-indicated.

1. Use of Open Carius Tubes

In the micro-estimation of chlorine in organic materials such as foodstuffs and biological products, in which the chlorine is almost exclusively present in ionised form, the open Carius tube technique can with advantage be used to destroy organic matter. This procedure, in effect, consists of adding to the material fuming nitric acid and a known excess of silver nitrate, and heating in a long test-tube, whilst passing through the liquid a steady stream of fine air bubbles. To complete oxidation, a few milligrams of persulphate are added and the heating is continued until all organic matter has been destroyed. The chloride is immediately fixed as the insoluble silver salt, and so volatilisation of chlorine is prevented.* The excess of silver is then estimated by the Volhard technique.

2. Sulphuric/Nitric Acid Oxidation

Oxidation in a Kjeldahl flask with sulphuric and nitric acids is recommended as a preliminary process in the estimation of traces of heavy metals in organic matter. Typical examples are afforded by the estimations of arsenic, antimony, mercury, lead, etc., in foodstuffs or in biological material. The weighed sample is first heated gently with nitric acid until a clear solution results, and then sulphuric acid is added and the heating is continued until all organic matter has been destroyed.

If much chloride is present in the material, the loss of certain metals, e.g. arsenic or antimony, may easily occur unless stringent precautions

* Smirk, F., *Biochem. J.*, 1927, **21**, 31.

are observed. These metals form chlorides, and some organo-metallic compounds, which are quite volatile if in the reduced condition. When organic matter is present, conditions of reduction may easily prevail locally, even though an excess of nitric acid is present in the flask. It is therefore expedient to avoid charring of the organic matter by using nitric acid alone until all chloride has been removed, probably as nitrosyl chloride. Then the sulphuric acid may be added and the oxidation allowed to proceed at a more vigorous rate.

Phosphorus may be estimated in the resultant liquid from sulphuric/nitric oxidation, either by Neumann's volumetric method (p. 164) if the quantities are larger than 0·5 mg. or colorimetrically after reduction of phospho-molybdate (p. 314) if the quantities are less than this. In the latter case it is essential that all nitric acid should be removed, and in consequence other oxidising agents are usually preferable.

3. Perchloric Acid Oxidation

With the advent of cheap supplies of this acid in concentrated form its use as an oxidising agent has been widely advertised. It possesses the virtue of having a boiling-point almost as high as that of sulphuric acid, and in the presence of organic matter it has strong oxidative properties. It has therefore been recommended for the oxidation of blood, tissues, foods, etc., preparatory to carrying out micro-estimations of various metallic radicals.

Precautions should be exercised when using this acid, since under certain conditions oxidation takes place with explosive force. This disadvantage is to some extent minimised if perchloric acid be used as an adjunct to sulphuric acid. The amount of perchloric acid required is then minimised, being at most equal in volume to the bulk of sulphuric acid taken. If a preliminary digestion with sulphuric acid is made, the risk of explosion is greatly reduced.

Sulphuric/nitric/perchloric acid oxidation has also been advocated,* since it has been found that a small amount of nitric acid has a catalytic effect upon the oxidation process. Mixed nitric/perchloric acid oxidation has been suggested for the micro-analysis of sulphur compounds.†

4. Oxidation with Nitric Acid and Ammonium Nitrate‡

In the micro-analysis of biological materials for trace elements, when the bulk of the organic matter to be destroyed is very great in comparison with the micro quantities of metals to be estimated, the relatively vast amounts of strong acids required to ensure complete oxidation result in the production of a final solution containing a large excess of acids. For some estimations this is a disadvantage, for the acid has to be neutralised

* Kahane, E., *Bull. Soc. Chem. Biol.*, 1932, **14**, 294.
† Lematte, L., Boinot, G., and Kahane, E., *Compt. Rend. Soc. Biol.*, 1927, **96**, 1211.
‡ Milton, R., Hoskins, J., and Jackman, W., *Analyst*, 1944, **69**, 299.

or volatilised off, and the latter process is sometimes inadvisable. A more recent suggestion for oxidation* is to use a mixture of nitric acid and ammonium nitrate. This method has been worked out particularly for blood and urine estimations, and allows of the estimation on one sample after oxidation of Ca, Mg, K, Na, Fe, P, and S.

The procedure is as follows. The sample to be oxidised is heated in a micro-Kjeldahl flask with nitric acid until frothing ceases. To this is then added a solution of 1 part of concentrated nitric acid and 4 parts water which has been treated with solid ammonium nitrate until no more dissolves. (This solution then contains about 50% of ammonium nitrate by weight.) Heating is continued very gently at first and finally quite vigorously until a clear colourless melt remains. The excess of ammonium nitrate is then removed by volatilisation and there is left a residue approximating to a true "ash."

Certain precautions are necessary when carrying out micro-analysis after wet oxidations. With nitric/perchloric acid oxidation of biological material in particular, there is a tendency to form certain intractable meta-phosphoric acid complexes, which not only do not give the usual tests for phosphates but which may also inhibit the precipitation of other substances, such as sulphate with barium chloride and calcium with oxalate.

It is essential after these oxidations that the residue should be boiled for some time with dilute hydrochloric acid, to hydrolyse these complexes completely, before proceeding to subsequent micro-analyses.

* Milton, R., Hoskins, J., and Jackman, W., *Analyst*, 1944, **69**, 299.

THE DETERMINATION OF MOLECULAR WEIGHTS

WHILST molecular weight determination by the ebullioscopic method can be carried out on the semi-micro scale by the use of small-scale apparatus of conventional design,* this procedure is not so successful on the milligram scale on account of the technical difficulty of reducing the size of accurate Beckmann thermometers. The differential thermometer of Menzies and Wright† is as yet the most promising instrument for small-scale ebullioscopic work.

In contrast one can, by using Rast's method, determine the molecular weight of a substance from its depression of the freezing-point of camphor, or a few other substances which are listed below, with the expenditure of only 0·1–0·5 mg. of material. The success of this method depends both on the high solvent power of camphor for most organic substances and on the fact that its Raoult constant for the molecular depression of the freezing-point is so high ($K=40$) that 10% mixtures of camphor with other substances have melting-points lowered by 5°–20° C. Thus with an accurate thermometer of ordinary type, calibrated to fifths of a degree, one can, by determining the melting-point of about 5 mg. of a mixture in a capillary tube, obtain a reliable figure for a molecular weight.

Barger's method of comparing vapour pressures of bubbles in capillary tubes, though not accurate, is also useful in many cases.

The apparent molecular weights of small quantities of complex substances can, of course, be carried out by special methods, e.g. by determination of sedimentation velocity, specific viscosity, or osmotic pressure, or by measuring with X-rays the dimensions of the crystallographic "unit cell," but these involved techniques cannot be discussed in this volume.

Procedure for Determination of Molecular Weights by Rast's Methods ‡

1. *Purification and Testing of the Solvent.* Camphor for molecular weight determination should be purified by crystallisation or sublimation until its melting-point is 176°–180° C. A small stock (10–20 g.) should be ground to a powder in an agate mortar (moistening with ether to prevent

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* Apparatus suitable for working with 10–20 mg. of material has been described by Pregl ("Quant. Org. Mikroanalysis"); Rieche (*Ber.*, 1926, **59**, 218; *Mikrochem.*, 1933, **12**, 129), and Bobranski and Sucharda ("Semi-micro Methods for the Elementary Analysis of Organic Compounds," Gallenkamp, London, 1936), amongst others. Operational details are identical with those required for the corresponding macrochemical apparatus.

† Menzies, A. W. C., and Wright, S. L., *Proc. Nat. Acad. Sci.*, 1921, **7**, 77-80.

‡ Rast, *Ber.*, 1922, **55**, 1051, 3727.

sticking), dried thoroughly inside a vacuum desiccator between sheets of filter-paper, and kept in a wide-necked, glass-stoppered bottle. Before use it is essential to determine both the exact melting-point and also the Raoult constant, K (for calculation see below, p. 105), *with each batch of material*, using a pure substance such as acetanilide for the solute. In a few cases camphor reacts directly with organic substances (e.g. with some alkaloids and with a few organic acids), and when this is suspected the result should be checked by using another solvent. Other suitable solvents for the Rast procedure are:

> Camphene, m.p. 49° C., K=31.
>
> Bornylamine, m.p. 164° C., K=41.
>
> Cyclohexanol, m.p. 24·5° C., K=38.

2. *Charging of Melting-point Tubes.* Melting-point tubes are made by drawing out carefully cleaned quill-tubing to capillaries about 4 cm. long,
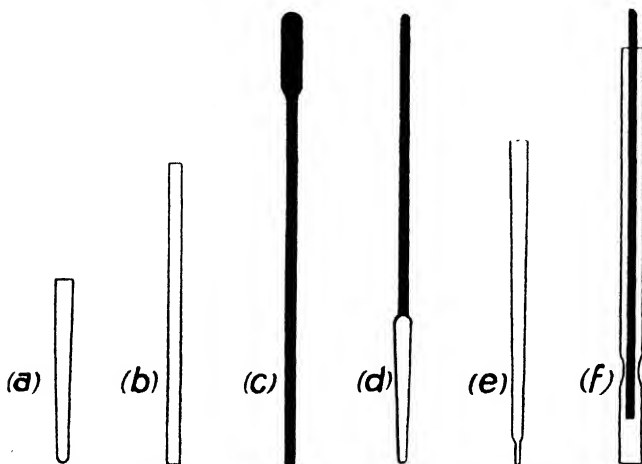
Fig. II.16   Filling of Capillary Tubes for Rast's Method of Molecular Weight Determination.

tapering in diameter from about 2 mm. at the sealed end to just over 3 mm. at the open end (see fig. II.16, *a*). Melting-point tubes are charged by means of narrower, untapered capillaries (fig. II.16, *b*) about 6 cm. long and 1–1·5 mm. diameter, which will fit snugly into the bottom of the wider tube *a*, together with thin glass rods (fig. II.16, *c*) which will slide evenly in the capillaries *b*.

Before charging, a tapered capillary *a* supported inside a small counter-poise bottle (fig. I.2 *b*, p. 16) is weighed on a micro-balance and then transferred to a copper block. The substance under investigation is powdered in a clean watch-glass and a small amount (0·1–0·5 mg.) is pressed up into tube *b*, which is then cleaned externally with a fine brush and carefully transferred to tube *a*. After ejecting the substance by means of the rod *c*, both *b* and *c* are removed, and tube *a*, in its supporting bottle,

is reweighed.   Camphor, or other solvent, in amount roughly 10 times that of the substance previously taken, is then introduced in a similar manner, using a fresh tube *b* and rod *c*, and the melting-point tube is again weighed. During the whole of this filling operation neither tube *a* nor its supporting bottle must be touched by hand.

The melting-point tube *a* is then sealed near its upper end in a blow-pipe flame, and the fused glass is drawn out to form a handle approximately 5 cm. long (fig. II.16, *d*).

Liquids may be filled into melting-point tubes by using somewhat narrower tapered tubes of which one end has been drawn out to a short hair capillary (fig. II.16, *e*).   After a trace of liquid has been sucked up, the filling tube is wiped externally and introduced carefully into the melting-point tube *a*, so that its tip does not touch the inner wall.   The drop of liquid is then blown out and the filling tube is withdrawn with equal care.

Viscous substances may be introduced into melting-point tubes by means of a narrow glass rod protected by a clean outer capillary tube (fig. II.16, *f*).

3. *The Determination of the Melting-point.* Before the accurate melting-point is determined, the contents of the melting-point tube are thoroughly mixed by immersing in a heating bath, maintained at a few degrees above the melting-point of the solvent, until the whole of the mixture has liquefied. During this operation the capillary should be held by the glass thread so that the hollow part is completely immersed.   The hot tube should be examined carefully with a lens to make sure that all the substance has dissolved; if it fails to do so, then another solvent should be used.

The capillary tube is then attached to a suitable short-range thermometer by means of a rubber band and placed in a standard melting-point apparatus. The temperature of the heating bath is raised at the usual rate until the solid contents of the melting-point tube change to a turbid mush of liquid and crystals.   The tube is then examined through a lens as the heating is continued very carefully, and the point at which the last crystal disappears is noted.   With care and practice this can be determined to within 0·2° C. Finally the tube should be allowed to cool and the melting-point should be redetermined until at least two concordant observations have been made.

4. *Calculation.* The Raoult constant for the freezing-point is first calculated, from the observations with a reference substance (p. 104), from the equation:

$$K = \frac{M.L.\varDelta t}{1000S}$$

where   M=molecular weight of substance taken, and S its weight,

L=weight of solvent (camphor, etc.),

$\varDelta t$=difference in melting-point between the pure solvent and the solution.

Therefrom the molecular weight of the unknown substance is computed from the formula:

$$M = \frac{1000K.S}{L.\Delta t}$$

where the symbols are the same as those given above.

Approximate Estimation of Molecular Weights by Barger's Method*

As long ago as 1904 it was shown by Barger that the molecular weight of any dissolved substance could be assessed, very approximately, by making use of the fact that the degree of lowering of the vapour pressure of a solution is dependent upon the total molar concentration of the dissolved particles.

If drops of a solution a of unknown molar concentration are drawn into a capillary tube (fig. II.17) and spaced with air between drops c of a solution of a known substance at a known concentration in the same solvent, then isothermal distillation can occur from drop a to drop c and *vice versa*. Drop a will increase in length if solution a is more concentrated than c, and inversely it will decrease if c is the stronger solution. Only when a and c

Fig. II.17.   Capillary Tube for Estimation of Molecular Weights by Barger's Method.

have identical molar concentrations should there be no change in the relative dimensions of the bubbles when the tube is stored over a long period. These changes can be observed by placing the capillary tube under a reading microscope and are appreciable in a few hours if a volatile solvent such as acetone is used, though with aqueous solutions it is advisable to wait for a few days before making a decision.

By selecting a number of reference solutions c and setting up at the same time a whole series of similar capillary tubes, one can in this way bracket the molar concentration of the known and unknown solutions, and so estimate the molecular weight of a dissolved substance to an accuracy of about 10%. It is useful to choose a stable coloured compound, such as azobenzene, as the reference substance, for then it is easy to differentiate between the alternate bubbles.

For use with organic solvents capillary tubes of about 1·0 mm. internal diameter are suitable, though with aqueous solutions the diameter should be larger (1·5–2 mm.). Drops of a liquid can easily be introduced into a capillary tube merely by dipping one end of it into the required solution, and the drops can be run along the tube by tilting. In all, about seven drops of liquid, with intermediate air spaces, should be introduced into

* Barger, *J. Chem. Soc.*, 1904, **85**, 286.

each tube, and the ends of the tubes should then be sealed in the flame of a micro-burner. The tubes should be mounted in soft wax on a microscope slide, and the bubble lengths can be recorded in terms of the scale of the microscope eyepiece.

Errors due to the wetting of the walls of the tube with the solutions concerned limit the accuracy of the method, and it is not advisable to place reliance upon just one or two observations of bubble-lengths. It takes little time and effort, however, to prepare several series of capillary tubes.

Barger's method of estimating approximate molecular weights is not to be recommended when other procedures can be used. It has, however, one definite merit: it can be used with substances which are soluble only in water and with ionised compounds, such as the salts of organic acids. When salts are being examined, the reference substance should be a simple salt of similar ionic character, with which it can be assumed that the degree of dissociation varies with dilution in the same way. Thus sodium acetate could be taken as the comparison substance for the sodium salt of a mono-basic acid, and sodium oxalate for comparison with a salt of a dibasic acid. Sodium picrate is also a convenient reference substance. Dyestuffs should, in general, be avoided as reference compounds, since many of them are double salts, whilst others, though crystalline, may be contaminated with inorganic salts.

SPECIAL PROCEDURES FOR THE
ESTIMATION OF ORGANIC GROUPS

A. The Determination of Acetyl Groups

PRINCIPLES. *Acetyl groups* attached to either oxygen (R—O—COCH$_3$) or nitrogen (R$_2$N—COCH$_3$) can be determined micro-chemically by Perkin's method—hydrolysis followed by quantitative distillation of the resulting acetic acid—following the procedure of Kuhn and Roth.*

Benzoyl groups (—COC$_6$H$_5$) can be estimated by the same method.

Since acetylation and benzoylation are both convenient methods for characterising —OH and —NH groups, it follows that acetyl group determination can be a valuable aid in the micro-analysis of *alcohols, phenols, amines,* and *imines.*

By introducing a preliminary oxidation with chromic acid, Kuhn[†] has been able to apply the procedure to the determination of *ethoxy groups* (—O—C$_2$H$_5$, N—C$_2$H$_5$) and to the estimation of *alkyl side chains* in many types of organic molecules, including terpenes and compounds of the sterol group.

The analysis is not one that can be carried out successfully by following a rigid procedure, since (i) the formation of volatile acidic products may invalidate the whole analysis, and (ii) the ease of hydrolysis of an acetyl derivative depends both on its structure and on its solubility in the chosen hydrolysing agent. Because of the inherent difficulties, analysis in duplicate is therefore essential.

The Kuhn-Roth procedure which is described below involves the distillation of acetic acid as such. In Perkin's macro-chemical method the acetic acid is removed in the form of ethyl acetate by distillation with alcohol and subsequently saponified with standard alkali. Its chief drawbacks are the loss of volatile ester and the risk of oxidation of the alcohol to acidic products. Recently, however, Wiesenberger[‡] has developed this method on the micro-chemical scale. He uses methyl alcohol for the distillation and hydrolyses the substances by means of *p*-toluene-sulphonic acid (see below). This procedure minimises errors due to charring, but very great care has to be taken to prevent loss of the volatile ester, which must be collected and saponified in a closed receiver. For this reason only the more adequately tested method is described in detail.

REAGENTS FOR THE HYDROLYSIS. The hydrolysing reagent should, if possible, dissolve the substance to be analysed and effect its quantitative

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* Kuhn and Roth, *Ber.*, 1933, **66**, 1274.
† Ibid.; also Kuhn and l'Orsa, *Z. Angew. Chem.*, 1931, **44**, 847.
‡ Wiesenberger, E., *Mikrochem.*, 1942, **30**, 241.

hydrolysis in $\frac{1}{2}$ hour at 100° C. The complete hydrolysis of very stable compounds may, however, take up to 3 hours. Selection may be made from the following:

(a) *Sulphuric acid:* made by diluting 10 ml. of concentrated acid with 20 ml. of distilled water.

(b) *p-Toluene-sulphonic acid:* in 25% solution in water. With this there is no risk of formation of sulphur dioxide.

(c) *Sodium hydroxide:* this may be used either in 5N aqueous solution or in methyl alcohol which has previously been distilled from caustic soda. The latter reagent is the more suitable for the saponification of N-acetyl
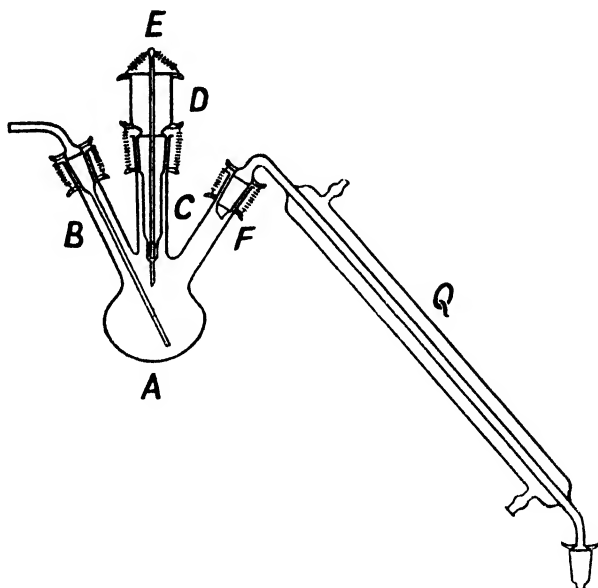
Fig. II.18.   Apparatus for Determination of Acetyl Groups.

groups.   Much weaker acids or alkalis can, of course, be used for the analysis of very sensitive compounds.

Sparingly soluble compounds should first be dissolved in pyridine (1 ml.) and then treated with methyl alcoholic caustic soda.

APPARATUS. The special apparatus devised by Kuhn and Roth for this analysis is shown in fig. II.18.*   The three-necked reaction flask *A* has a capacity of 60 ml.   Limb *B* terminates with a B10 socket-joint for the insertion of the gas inlet-tube, which reaches to the bottom of the flask.   To this are connected a bubble-counter, filled with 50% caustic potash solution, and a U-tube filled with soda-lime granules (e.g. Ascarite).

* As an alternative the apparatus of E. Wiesenberger (*Mikrochem.*, 1947, **33**, 51) may be used.

The centre limb C supports a funnel D of 8 ml. capacity, which is closed by a ground-in glass rod E. The third limb F leads upwards at an angle of about 50° from the centre limb and terminates in a B10 socket. To this is connected, by a spring-fitted ground joint, a quartz condenser Q, of which the central tube, about 40 cm. long, is bent through an angle of 40° at a distance of about 5 cm. from the joint. Thus the condenser can be either turned vertically upwards for refluxion or inclined downwards for distillation.

It is important to moisten the ground joints with distilled water and not to lubricate them with grease or phosphoric acid.

PROCEDURE FOR HYDROLYSIS. Weigh the substance (5-10 mg.) into the flask from a long-handled weighing-tube (p. 17) or small boat. In the case of a liquid, use a capillary tube and break it inside the flask by pressing on the solid handle with a glass rod, rinsing in this with a few drops of the hydrolysing reagent.

Then add the hydrolysing reagent from a pipette, close the funnel at E, and seal the joint with 1–2 ml. of water. For acid hydrolyses (a or b) use 1 ml. of the reagent; for alkaline hydrolyses use 1 ml. of $5N$ aqueous alkali or 4 ml. of alcoholic alkali.

Fit condenser Q in the vertical position, and pass nitrogen through the reaction flask, from B, at the rate of about 50 bubbles per minute.

Place the reaction flask in a large beaker of boiling water so that the side limbs B and F are covered, and keep it at the boiling-point for the required time ($\frac{1}{2}$ hour minimum). Then remove the water-bath, allow the flask to cool, open the seal at E, and wash down the reflux condenser with about 5 ml. of water from a wash-bottle.

PROCEDURE FOR DISTILLATION. Turn the condenser into the distillation position and place under it, as a receiver, a 25 ml. measuring cylinder of Pyrex or Jena glass which has previously been cleaned and then steamed out till alkali-free (compare p. 81). Replace the glass rod E and again seal this joint with a few drops of water.

If methyl alcohol has been used for the hydrolysis or pyridine as the solvent, distil this over before proceeding further. About 5 ml. of distillate should be collected, and the receiver should then be washed out again with distilled water.

To the flask then add, through funnel D, the following:

    (a) after hydrolysis by sulphuric acid—1 ml. of $5N$ caustic soda;

    (b) after hydrolysis by p-toluene-sulphonic acid—0·5 ml. of $5N$ caustic soda;

    (c) after hydrolysis by alkali—1 ml. of sulphuric acid reagent a (above).

Place one or two pieces of pumice in the flask, rinse down the funnel with

distilled water, refit the rod E, and then fill funnel D almost to the top with distilled water (about 5 ml.).

Then distil the mixture in flask A, using a small flame and an asbestos gauze, and adjusting the rate of heating so that about 1 ml. of distillate collects per minute. After collecting about 5 ml. of distillate, when the liquid in the flask should have been reduced to about 4 ml., run in slowly, without interrupting the distillation, the 5 ml. of water in the funnel D. Reclose E and refill the funnel.

Continue this procedure until 20 ml. of distillate have been collected. Then pour the contents of the cylinder into a clean, steamed-out flask, taking care not to lose any drops of liquid from the end of the condenser. Replace the cylinder and continue the distillation as before, collecting a further 15 ml. of distillate. Titrate each 15–20 ml. portion of the distillate until no more acetic acid distils over.*

PROCEDURE FOR TITRATION. Test each portion of the distillate for the absence of sulphate (indicative of decomposition of the sulphuric acid) by adding a few drops of barium chloride solution and boiling for 7–10 seconds to expel carbon dioxide. If, after cooling, the solution is turbid, then the whole analysis must be rejected.

Then add a drop of phenolphthalein indicator and titrate with $N/100$ sodium hydroxide or baryta.

B. The Determination of Methyl Groups attached to Carbon

Kuhn and Roth† have shown that the oxidation of hydrocarbon chains, CH_3—CH_2—CH..., with chromic/sulphuric acid mixture usually proceeds until an equivalent amount of acetic acid is produced, the acetic acid not being oxidised further. Consequently side-chain methyl groups attached to carbon, i.e. CH_3—C, can often be estimated quantitatively by combining chromic acid oxidation with acetic acid distillation as described above (pp. 108–111). The Kuhn-Roth procedure is generally applicable to aliphatic compounds, though side-chain methyl groups do not give quantitative yields of acetic acid (i) when attached directly to an aromatic ring, as in toluene or (ii) when *gem* dimethyl or *tert*-butyl groups, $(CH_3)_2CH\cdot$, $(CH_3)_3C\cdot$, are present, since these yield acetone; and consequently the results of this analysis must be interpreted with discrimination.

A useful application of this method is the differentiation between *ethoxyl* and *methoxyl* groups, for ethoxyl groups can be oxidised quantitatively to acetic acid, whereas methoxyl groups are completely destroyed by chromic acid.

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* The fractional distillation procedure given above minimises the chances of error due to overheating of the liquid in the flask. The collection of four successive fractions is usually sufficient.

† Kuhn and Roth, *Ber.*, 1933, **66**, 1274.

PROCEDURE. The oxidation is carried out either in the apparatus of fig. II.18 or, for volatile substances, in a bomb-tube.

For oxidation in the open flask, treat the weighed substance (5–10 mg.) with 5 ml. of a mixture prepared from 20 ml. of $5N$ chromium trioxide crystals (A.R.) in water and 5 ml. of concentrated sulphuric acid and reflux for 2 hours.

If a bomb-tube is used, weigh the substance in a sealed capillary tube, introduce 5 ml. of the chromic/sulphuric mixture (above), seal the bomb-tube, heat for 2 hours at 120°, and then open the tube and wash its contents into the distillation flask.

When the oxidation is complete, cool the mixture under a tap and reduce the excess of chromic acid solution by adding, drop by drop, a dilute solution of hydrazine until the brown solution *just* changes to green. Do not use an excess of hydrazine. Then add 6 ml. of $5N$ caustic soda and 1 ml. of phosphoric acid (sp. gr. 1·7), rinse down the condenser tube, and carry out the quantitative distillation of the acetic acid as described on pp. 110–111.

C. The Determination of Methyl or Ethyl Groups attached to Oxygen, Nitrogen, or Sulphur (Micro-Zeisel Estimation)

PRINCIPLES. On the micro-chemical scale, the Zeisel method for the estimation of *methoxyl* groups by heating an organic substance with hydriodic acid and estimating the evolved methyl iodide by precipitation with silver nitrate was successfully developed by Pregl, who also applied his procedure to Herzig and Meyer's method for estimating $N-CH_3$ groups. For the latter analysis Friedrich[*] has modified the apparatus still further so that simple alkylimino groups and also certain alkyl groups attached to sulphur can be determined.

Ethoxyl $(O-C_2H_5)$ and *ethylimino* $(N-C_2H_5)$ groups can also be estimated by the Zeisel method, but higher alkyl groups do not give sufficiently volatile iodides. It is possible to differentiate between the volatile methyl and ethyl iodides by absorption in alcoholic trimethylamine,[†] for whilst tetramethyl-ammonium iodide is insoluble in absolute alcohol, trimethyl-ethyl-ammonium iodide dissolves.

Alternatively, $O-C_2H_5$ or $N-C_2H_5$ groups may be estimated separately by the chromic acid oxidation method of p. 111.

The alkyl halides liberated by the Zeisel decomposition can be estimated by:

(i) Collection in alcoholic silver nitrate and gravimetric determination as silver iodide.

(ii) Absorption in pyridine and volumetric estimation as pyridinium iodide, either by Volhard's method with thiocyanate or by means of mercuric oxycyanide[‡] (p. 87).

* Friedrich, A., *Mikrochem.*, 1929, **7**, 195.
† Küster, W., and Maag, W., *Z. Physiol. Chem.*, 1923, **127**, 190.
‡ Ingram, G., *Analyst*, 1944, **69**, 269.

(iii) Absorption in a solution of bromine in sodium acetate/acetic acid and conversion to iodic acid (pp. 89–90).

Inconsistent and usually low results can frequently be ascribed to incomplete decomposition of the organic substance by the hydriodic acid. In alkyloxy group determinations the fault can usually be traced to incomplete dissolution of the substance in the hydriodic acid, but this trouble can often be overcome by dissolving the reactant in a mixture of phenol and acetic anhydride, or, still better, propionic anhydride (see "Procedure," below).

The determination of N-alkyl or S-alkyl groups is much more difficult, and repeated distillation from hydriodic acid may be needed before the fission of the alkyl group is complete.

Apparatus for Estimation of O—CH₃ or O—C₂H₅ Groups (fig. II.19)

Fig. II.19. Apparatus for Determination of Alkyloxy Groups by Zeisel's Method.

The apparatus for micro-Zeisel estimations, shown in fig. II.19, is best made in one piece from Jena or Pyrex tubing of 5 mm. bore. The reaction flask A, of 4–5 ml. capacity, is fitted with a side-arm for connection to a carbon dioxide generator. If a Kipp is used for this purpose then the gas should be passed through a bubbler containing sodium carbonate solution in order to remove traces of hydrochloric acid spray.

The volatile reaction products from A ascend through the water condenser B^*, and pass through the gas washer C, and splash bulb to a receiver.

For gravimetric estimation of silver iodide, the receiver shown at R is convenient, whilst for absorption in pyridine the design S is more suitable. Inside S the gases pass down the central delivery-tube T, issuing at the bottom in small bubbles which ascend slowly through the absorbent. This fills the space between the glass spiral and the outer jacket, which, to facilitate drainage, is fitted with a stopcock.

The micro-Zeisel apparatus should be clamped, between split corks K, at the top of the condenser tube, and should be placed at such a height that

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* Cf. Elek, *Ind. Eng. Chem.* (Anal. Edn.), 1939, **11**, 174.

the absorption apparatus can easily be lowered away for rinsing down and removal.

The gas washer C serves as a trap for the removal of iodine vapour, and should be charged with 1 ml. of 5% cadmium sulphate solution containing a suspension of red phosphorus.*

PROCEDURE FOR DECOMPOSITION WITH HYDRIODIC ACID

1. Perfect results cannot be obtained unless the whole apparatus is absolutely clean, dry, and *alcohol-free*. It should be cleaned in chromic/sulphuric acid mixture, rinsed in distilled water, then *acetone*, and dried by warming, with suction, over a flame.

2. Solids easily soluble in phenol/propionic anhydride mixture (see below) should be crushed in an agate mortar and weighed into small tinfoil cups of about 10 mg. weight. These are made by shaping a circle of tinfoil, of about 1 cm. diameter, about the end of a glass rod. Each cup should be examined for holes, brushed, and weighed. After inserting the powdered substance (3 to 7 mg.), the top of the cup should be closed together, by pressing the foil with forceps, before reweighing.

Solids which dissolve with difficulty should be weighed directly into the reaction flask from a long-handled weighing-tube (p. 17) and the tinfoil added separately. The latter yields stannous iodide, which prevents bumping and promotes even boiling of the hydriodic acid.

Liquids and hygroscopic substances should be weighed into small glass cups, of about 4 mm. diameter, fitted with ground stoppers.   ·

3. When the gas washer and the absorption apparatus have been filled (for alternative absorbent solutions, see below), the foil containing the sample is introduced into the flask A with forceps, and 3–4 small crystals of phenol and 6-8 drops of propionic anhydride are then added. If a difficultly soluble substance is being analysed, the flask is warmed with a low flame of a micro-burner to effect solution. If the substance dissolves in hot hydriodic acid then the addition of the above solvents is unnecessary.

The constriction W at the top of absorption-tube T is then sealed with a drop of water and a small bung is inserted at its upper end.

2 ml. of hydriodic acid, of purest M.A.R. quality (sp. gr. 1·7), is then added to the reaction flask from a clean pipette and the carbon dioxide generator is connected to the side-arm. The flow of gas is regulated by a screw clip so that the bubbles rise one at a time through the receiver.

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* Pregl recommended the use of red phosphorus only, but since iodine, hydriodic acid, and hydrogen sulphide may all be carried over during the distillation, this is insufficient.

Several workers have suggested that 5% sodium thiosulphate might be used in place of red phosphorus, but it has been shown[1] that this leads to low results, since the alkyl iodide in part dissolves and reacts with the thiosulphate.

[1] White, *Ind. Eng. Chem.* (Anal. Edn.), 1944, 16, 207.

4. The flask is heated gently with a low flame from a micro-burner protected by a draught chimney. As the foil dissolves, an acceleration of the gas current occurs, and bubbles rise more rapidly in the receiver, but on no account should the setting of the screw clip of the carbon dioxide lead be altered when the hydriodic acid commences to boil.

After 30 minutes the apparatus is raised with its clamp until the end of the delivery-tube is about 2 cm. above the liquid in the absorption vessel. *The burner is then removed* and the delivery-tube is washed down inside and outside with water. Alcohol should be used if the gravimetric procedure is being followed, and in this case adhering particles of silver iodide may be removed with the help of a "feather."

Since the reactivities of organic compounds differ so widely it is advisable to carry out this decomposition in duplicate, and to allow a period of at least 45 minutes for the decomposition in the second instance.

GRAVIMETRIC METHOD OF ANALYSIS—ESTIMATION AS SILVER IODIDE

If the alkyl iodide is being estimated gravimetrically by conversion to silver iodide, the absorber shown at R should be used.

Alcoholic silver nitrate reagent is prepared by dissolving 4 g. of silver nitrate in 100 g. of 95% alcohol by refluxing for 4 hours on a water-bath. Since fresh solutions give irregular amounts of silver iodide, the mixture should be kept for 2 days, decanted from the separated silver into a brown glass bottle, and then "aged" by storage for at least a week. Even after taking these precautions it is necessary to add an empirical correction of 0·06 mg. per ml. of silver nitrate solution to the recorded weight of silver iodide to compensate for incomplete precipitation.

When the decomposition with hydriodic acid (section above) has been completed and all the reaction product has been washed down into an absorption-tube, the double compound $AgI,AgNO_3$ is destroyed by adding 5 drops of nitric acid and heating the tube in a water-bath until its contents begin to boil and all the silver iodide settles at the bottom. This usually takes about 2 minutes. In strongly alcoholic solution the decomposition takes a longer time. If it is not complete then high results will be obtained.

When the mixture has cooled, the precipitate is siphoned off into a weighed filter-tube, as described on pp. 22–23, washed with distilled water and alcohol, dried at 110° C., and weighed. The empirical correction is then added before the result is calculated: 1 mg. of $AgI \equiv 0·1321$ mg. of OCH_3 or 0·1918 mg. of OC_2H_5.

VOLUMETRIC METHOD USING IODINE TITRATION

The absorber R is used and the receiver is charged with 2 ml. of a 10% solution of sodium acetate in glacial acetic acid to which has been added 6–8 drops of bromine.* To prevent bromine vapour from escaping into

* Cf. p. 89.

the laboratory, the mouth of the receiver is plugged with cotton-wool moistened with formic acid. If the solution becomes decolorised during the distillation then 3–4 drops more of bromine should be added.

After completion of the distillation, the contents of the absorber are rinsed into a 150 ml. conical flask containing 5 ml. of sodium acetate solution. Excess of bromine is destroyed with formic acid, and the titration is completed by the Leipert procedure for the estimation of iodine (pp. 89–90).

1 ml. of $N/50$ thiosulphate $\equiv 0 \cdot 1034$ mg. of OCH_3 or $0 \cdot 1501$ mg. of OC_2H_5.

VOLUMETRIC ANALYSIS WITH MERCURIC OXYCYANIDE

Absorber S is filled to the top of the spiral with redistilled A.R. pyridine. On completion of the distillation, the contents of the spiral-fitted tube S
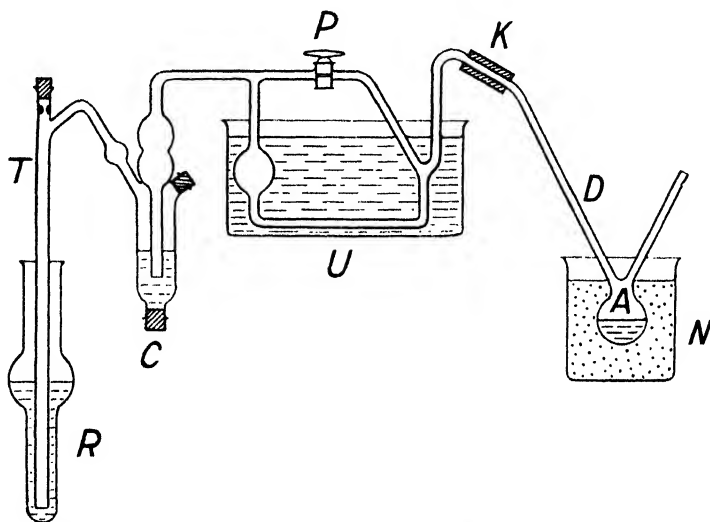
Fig. II.20.   Apparatus for Determination of Methyl-imino Groups.

are washed into a 100 ml. round-bottomed flask fitted with an inlet-tube reaching half-way into the bulb and an outlet-tube for connection to a water-pump. The pyridine is distilled off under reduced pressure on a water-bath, and the residue of alkyl pyridinium iodide is then dissolved in 5 ml. of distilled water. 10 ml. of neutral mercuric oxycyanide reagent are then added, and the liberated base is titrated with $N/100$ sulphuric acid (compare pp. 87–88).

Estimation of Methyl or Ethyl Groups attached to Nitrogen or Sulphur

APPARATUS. The apparatus (fig. II.20) devised by Friedrich for the estimation of N—CH_3 groups differs from that of fig. II.19 in that a U-tube U and a by-pass P provided with a two-way stopcock are placed between the gas washer and the reaction flask, whilst an air condenser D is used in place of

a water condenser. Consequently hydriodic acid can be distilled and collected in the **U**-tube, which is immersed in a beaker of water at 90° C., without interrupting the flow of carbon dioxide through the whole apparatus.

Stopcock *P* is bored so that by turning it through 90° the flow of carbon dioxide can be cut off and the absorption side of the apparatus placed in communication with the air. In this way the hydriodic acid which collects in the **U**-tube during the heating may be sucked back into the reaction vessel without danger of disturbing the contents of either the gas washer or the final absorber. Thus the distillation of the substance with hydriodic acid may be repeated until the fission of the alkyl group is complete.

It is necessary to heat the reaction flask to 300°–350° C. This is carried out by immersing it inside a copper or nickel crucible *N* packed with copper oxide and holding a 360° thermometer.

METHOD.

1. The success of the analysis depends upon the complete dissolution of the substance in the hydriodic acid. Solids should be powdered in an agate mortar, weighed, and transferred to the reaction flask from a long-handled weighing-tube, and dissolved by warming in phenol/propionic anhydride mixture (p. 114) before adding the other reagents. These are: (i) 60–100 mg. of ammonium iodide (approximately 20 times the weight of substance taken); (ii) 1–2 drops of 5% gold chloride solution; and (iii) 2 ml. of hydriodic acid (sp. gr. 1·7).

The iodimetric method of p. 89 is to be recommended for the analysis of the resultant alkyl iodide.

2. When all the reagents have been added and the carbon dioxide stream has been regulated as in the Zeisel determination (p. 114), flask *A* is placed inside its crucible heater and warmed gradually up to 160° C. with the small flame of a Bunsen burner. The temperature is then raised more slowly so that it reaches 200° C. in a further 20 minutes, during which time hydriodic acid gradually distils over into the **U**-tube, which is kept at 90° C. Finally the copper oxide bath *N* is heated up to 350°–360° C. with a larger flame and is kept at this temperature for 30 minutes more.

3. The flame is then removed, and, whilst maintaining continually the carbon dioxide stream, the receiver is lowered and the delivery-tube is rinsed down with water. The contents of the receiver *R* are removed for titration and replaced by a fresh batch of absorbent solution.

The contents of the gas washer *C* is examined, and if this is highly coloured it is replaced by a fresh solution also.

4. The distilled hydriodic acid is then transferred back to the reaction flask by attaching a rubber tube to the gas inlet and applying gentle suction. At this stage the temperature of the heating bath should be below 100° C.

The delivery-tube *T* is finally re-inserted into the fresh absorbent solution and the distillation procedure is repeated.

Further distillations should be carried out until the final thiosulphate titre corresponds to an alkyl group content of not more than 0·5%, which is the limit of accuracy of the estimation.

1 ml. of $N/50$ thiosulphate $= 0·3005$ mg. of CH_3 or $0·5808$ mg. of OC_2H_5.

Simultaneous Estimation of O—CH_3 *and* N—CH_3 *Groups*

If compounds contain both O—CH_3 and N—CH_3 groups, then it is possible to estimate them independently. To do this, regulate the temperature of the first heating process to $140°$ C. and maintain the reaction flask at this temperature for 40 minutes before detaching the receiver. Under these conditions only the alkyl*oxy* group is split off. Alkyl groups liberated by subsequent, more drastic, heating may be presumed to come from *N*-alkyl groups.

D. The Determination of "Reactive Hydrogen"

PRINCIPLES. The method of Zerewitinoff for the determination of "reactive hydrogen" (i.e. OH in carbinols, enols, etc., NH in amines) in organic substances by the quantitative evolution of methane on reaction with methyl magnesium iodide has been developed on the micro-chemical scale by Roth,* but the analysis requires extreme care in technique and exact attention to experimental details.

High results will be obtained if either the solvent or any part of the apparatus contains any trace of moisture. Since oxygen reacts with the Grignard reagent, the whole determination must be carried out in an atmosphere of nitrogen; whilst again the incomplete removal of excess of methyl iodide will introduce a noticeable error, since its vapour tension is appreciable.

Low results will be obtained if the test solution does not dissolve completely in the chosen solvent.

In view of all these difficulties, it is essential to carry out a blank determination immediately prior to each analysis. Accuracy greater than $\pm 3\%$ is not to be expected.

APPARATUS. The apparatus shown to scale in fig. II.21 has been developed from that of Roth.

The main reaction vessel A, of about 15 ml. capacity, is fitted with a side-arm and a B14 ground joint leading to a small retort B, in which is placed about 2 ml. of the Grignard reagent. The ground stopper of A carries a capillary tube lead to the gas-burette D, and also an internally-sealed tube F whereby the whole apparatus can be swept out with pure dry nitrogen.†

* Roth, *Mikrochem.*, 1932, **11**, 140; cf. Roth's revised editions of Pregl's monograph, published 1937 and 1947.

† This apparatus is quite convenient for carrying out other gasometric analyses and can be considered as an alternative to the Van Slyke manometric apparatus described in Part VI.

The micro-burette D has a capacity of about 4 ml. and is graduated to 0·01 ml. Mercury is used as the manometric liquid and the whole burette is immersed in a wide tube of water W for maintaining constancy of temperature.

Since the volume of gas produced by the chemical reaction is but a small proportion of the cubic capacity of the apparatus, it is essential to see that all volume measurements are made at exactly the same temperature, and consequently flask A, both before and after the reaction, must be immersed in a large beaker of water. Great care must be taken to see that all glass joints are exact and that they have been greased properly and secured firmly with springs.

Before use, the glass apparatus should always be cleaned, dried in an oven at 110° C., and the ground joints lubricated with vaseline.

PREPARATION OF REAGENTS

1. *Nitrogen.* Cylinder nitrogen should be passed through a short length of heated combustion tubing packed with reduced copper, then washed through 50% caustic soda, and finally dried by passage first through a bubbler containing sulphuric acid and then through a **U**-tube packed with phosphorus pentoxide.

2. *The Grignard Reagent— Methyl Magnesium Iodide.* 50 g. of pure *iso*-amyl ether, dried and

Fig. II.21. Apparatus for Determination of "Reactive Hydrogen" by Zerewitinoff's Method.

distilled from sodium metal, 4·5 g. of cleaned magnesium turnings, and 18 g. of freshly distilled methyl iodide are placed together in a dry 150 ml. flask fitted with a reflux condenser and drying tube. Reaction is completed by heating on a water-bath. The condenser is then refitted for distillation and the excess of methyl iodide is removed by heating the solution for at least ½ hour at 100° C. in a slow stream of nitrogen.

The last traces of methyl iodide are removed by fitting to the flask, by a ground joint, a **T**-piece with taps for (i) a nitrogen lead and (ii) a pump connection, and then evacuating at 50° C. and sweeping out with dry nitrogen for several times in succession.

The Grignard reagent, if prepared carefully in this way, may conveniently be stored, in 2 ml. portions, in sealed test-tubes over nitrogen, and will keep almost indefinitely.

3. As *solvents*, anisole and pyridine are the most convenient to use. Anisole should be dried for 2 days over phosphorus pentoxide, and then distilled from sodium into dry test-tubes, in which it should be sealed off promptly in about 5 ml. portions for storage.

Pyridine, free from homologues, should be dried over sodium for a few hours, distilled under reduced pressure, and shaken in a stoppered bottle with lumps of ignited barium oxide. After a few days' further storage it is fit for use.

Anethole, xylene, and iso-amyl ether are alternative solvents, of less general applicability on account of their inferior solvent powers.

4. *Substances for analysis* should be dried for not less than 1 hour over phosphorus pentoxide *in vacuo*, with heating if this is permissible (for apparatus, see p. 27).

PROCEDURE FOR ANALYSIS

(i) Dry the reaction vessel in an oven, and, whilst it is cooling, weigh out 3–5 mg. of substance, using for a solid a long-handled weighing-tube and for a liquid either a micro weighing-bottle or a capillary tube. After placing the weighed substance into the cooled reaction vessel, add from a pipette which has been oven-dried and cooled in a stream of dry nitrogen either 3 ml. of anisole or 1 ml. of dry pyridine. Fit together the whole assembly, sweep it out with a stream of dry nitrogen, and if necessary warm flask A till the substance dissolves.

Turn stopcock S so that connection between the gas-burette and the reaction vessel is broken, unstopper the small retort B and add to it, from a pipette which has been dried and cooled in nitrogen, about 0·5 ml. of the prepared Grignard reagent. Then replace the stopper and check that all the glass joints are secure.

(ii) Immerse the whole of the reaction vessel in a beaker of water *at room temperature*,* pass a slow stream of nitrogen through the whole apparatus for about 5 minutes, adjust the gas-burette to the zero mark, and then turn stopcocks S and T so that the nitrogen stream is disconnected and connection is made between the reaction flask and the gas-burette.

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* The water used to surround the reaction vessel should be drawn from the same stock tank as that used for filling the jacket of the gas-burette. Water drawn directly from the tap should not be used, as it will not be at air temperature.

After a few minutes test for any pressure change, and if necessary readjust, by stopcock S, to atmospheric pressure.   Read accurately the initial volume of the gas-burette.

(iii) Remove the beaker of water and mix the Grignard reagent with the substance by turning the bulb of the retort upwards.   Shake the reaction vessel and lower the levelling bulb of the gas-burette to keep the gases at about atmospheric pressure.   If necessary, warm the reaction vessel in a beaker of hot water.*

(iv) After 5 minutes re-immerse the reaction vessel in water at room temperature, readjust the pressure levels in the gas-burette, and read off the gas volume, to 0·005 ml., as soon as the pressure has become constant.

* Most reactions in pyridine solution are complete at room temperature; reactions in anisole may need heating up to 95° C.

TABLE OF MICRO-CHEMICAL METHODS FOR THE ESTIMATION OF ORGANIC GROUPS

Group	Structural Formula	Method of Analysis	References
Acetyl.	—O—COCH$_3$. N—COCH$_3$.	Hydrolyse and distil off acetic acid (or ethyl acetate).	See pp. 108–111.
Alcohols.	C——OH.	(i) Estimate "reactive hydrogen." (ii) Acetylate with acetyl chloride or acetic anhydride in pyridine and estimate excess of reagent used (not quantitative with tertiary alcohols).	See pp. 118–121. Stodola, *Mikrochem.*, 1937, **21**, 180. Petersen, J. W., Hedberg, K. W., and Christensen, B. E., *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15**, 225. Smith, D. M., and Bryant, W. D. M., *J.A.C.S.*, 1935, **57**, 61.
		(iii) Acetylate or benzoylate, isolate product and determine the acyl group. (iv) Colorimetric procedures for Methyl alcohol. Ethyl alcohol. Amyl alcohol. Glycerol.	See Part IV. p. 317. p. 318. p. 318. p. 319.
Aldehyde.	CH = O.	(i) Precipitate with dimedone and weigh (simpler aliphatic aldehydes only). (ii) Oxidise quantitatively with Benedict's solution. (iii) Oxidise with alkaline ferricyanide, titrating excess iodometrically. (iv) Titrate bisulphite compound. Volumetric methods (ii)–(iv) are applicable only to water-soluble aldehydes.	See pp. 332–334. See Glucose Estimation, Part III, p. 209. Compare estimation of lactic acid, Part III, p. 210.
Aldehyde.		For colorimetric methods for aldehydes and sugars, see Part IV. Acetaldehyde (compare also lactic acid). Acrylic aldehyde. Furfuraldehyde. Citral. Epihydrinaldehyde.	See pp. 210 and 322. See p. 322. See p. 322. See p. 321. See p. 323.
Alkyl.	C·(CH$_2$)$_n$·CH$_3$.	Oxidise with chromic acid to acetic acid and distil (estimation of terminal methyl groups).	K u h n and R o t h, *Ber.*, 1933, **66**, 1274; see pp. 112–113.

TABLE OF MICRO-CHEMICAL METHODS FOR THE
ESTIMATION OF ORGANIC GROUPS—Continued

Group	Structural Formula	Method of Analysis	References
Alkyl-imino.		See methyl-imino.	
Alkyl-oxy.		See methoxy.	
Amide.	—CO—NH$_2$.	(i) Hydrolyse with alkali and estimate ammonia, or titrate excess of reagent.	
		(ii) Decompose with nitrous acid and measure nitrogen (for water-soluble amides only).	See Part VI, pp. 558–561. Van Slyke, *J. Biol. Chem.*, 1929, **83**, 425.
Amino.	—NH$_2$.	*General methods for all bases.*	
		(i) Estimation of Pt in B$_2$,H$_2$PtCl$_6$.	See p. 96.
		Estimation of Au in B,HAuCl$_4$.	See p. 96.
		Estimation of Hg in double salt.	See pp. 98–99.
		(ii) Titration of chloride anion in the purified hydrochloride (this method is limited by the difficulty of preparing the pure salt).	For volumetric methods see Part II, p. 87; Part III, pp. 180–184. For electrometric methods see Part V.
		(iii) Direct titration of a salt with an indicator sensitive at low pH (accuracy and sensitivity both poor).	Compare Part III, pp. 163–164.
		(iv) Estimation of nitrogen by Kjeldahl's method.	See pp. 77–81.
Amino primary aliphatic.	Alkyl-NH$_2$.	(i) Decompose with nitrous acid and measure nitrogen.	Van Slyke, *J. Biol. Chem.*, 1929, **83**, 425. Part VI, pp. 558–561.
		(ii) Acetylate and estimate N—COCH$_3$, or the excess of the reagent used.	See *Acetyl group* and *Alcohols.*
Amino primary aromatic.	Aryl-NH$_2$.	(i) Diazotise, couple with H-acid, β-naphthol, etc., and estimate colorimetrically.	Compare Part IV, pp. 339, 344, 356.
		(ii) Brominate with bromide-bromate and titrate excess of bromine (for bases of known structure).	See uses of potassium bromate, Part III, pp. 175–176.
		(iii) Acetylate and estimate N—COCH$_3$.	See *Acetyl group.*
		(iv) Special colorimetric reactions— Part IV:	
		Aniline.	See p. 347.
		Sulphonamides.	See p. 356.
Amino secondary and tertiary.	R$_2$=NH R$_3$N.	Special colorimetric reactions:	
		Pyrrole.	See p. 355.
		Histidine.	See p. 351.
		Creatine and creatinine.	See p. 353.
		Tryptophane.	See p. 351.
		Nicotinic acid.	See p. 360.
		Adrenaline.	See p. 363.
		Indole and skatole.	See p. 369.
		Hexamethylene-tetramine.	See p. 356.
		Barbiturates.	See p. 331.

TABLE OF MICRO-CHEMICAL METHODS FOR THE ESTIMATION OF ORGANIC GROUPS—*Continued*

Group	Structural Formula	Method of Analysis	References
α-Amino-acids.	R—CH—NH$_2$ COOH.	(i) Add formaldehyde and titrate as a weak acid, using phenolphthalein.	Sörensen, *Biochem. Zeit.*, 1908, **7**, 45.
		(ii) React with ninhydrin and measure carbon dioxide evolved.	Part VI, pp. 556–558.
		(iii) React with nitrous acid and measure nitrogen evolved.	Van Slyke, *J. Biol. Chem.*, 1929, **83**, 425. Part VI, pp. 558 561.
		(iv) Precipitate with xanthydrol and weigh.	
		(v) Estimate colorimetrically with ninhydrin.	Part IV, p. 349.
Azo.	—N—N—.	(i) Reduce with titanous chloride or stannous chloride and back titrate reagent in excess (semi-micro only).	
		(ii) Polarographic reduction may be possible.	See Part V (compare p. 460).
		(iii) Known dyes are usually estimated colorimetrically.	See Part IV.
Benzene.	C$_6$H$_6$.	Dinitration and colour formation with butanone.	Part IV, p. 342.
Carbinol.		See alcohol.	
Carbo-hydrates.		(i) Titrate into Fehling's or Benedict's solutions using methylene blue indicator.	
		(ii) Titrate with alkaline ferricyanide.	See p. 209.
		(iii) Estimate colorimetrically: aldoses, ketoses, pentoses, rhamnose, etc., may be differentiated and determined separately.	See Part IV, pp. 331–338.
Carbonyl.	C O	See also aldehyde and ketone. (i) Reflux with hydroxylamine hydrochloride and titrate liberated acid, using methyl orange.	
		(ii) Precipitate 2 : 4-dinitrophenyl hydrazone and weigh.	
Carboxyl (organic acids).	COOH	(i) Titrate with sodium hydroxide or baryta in water or dilute alcohol, using phenolphthalein.	
		(ii) Dissolve in excess of alkali and back titrate.	
		(iii) Estimate silver in the silver salt.	
		(iv) React with bicarbonate in a Warburg apparatus.	See Part VI, pp. 562–573.
Carboxyl.	COOH.	(v) Special colorimetric procedures: Lactic acid. Pyruvic acid. Tartaric acid. Citric acid.	See Part IV. p. 324. p. 325. p. 326. p. 327.

TABLE OF MICRO-CHEMICAL METHODS FOR THE
ESTIMATION OF ORGANIC GROUPS—Continued

Group	Structural Formula	Method of Analysis	References
		Aceto-acetic acid.	p. 328.
		Benzoic acid, cinnamic acid.	p. 328.
		Hippuric acid.	
		Salicylic acid.	p. 329.
		Glycuronic acid.	p. 330.
		Tannic and Gallic acids.	p. 330.
Diazo.	Aryl-N_2X.	Couple with H-acid, β-naphthol, etc. and estimate colorimetrically.	Compare Part IV, pp. 338, 356.
Ester.	RCO—OR′.	Saponify with excess alkali and back titrate, using phenolphthalein.	
Ethylimino.	N—C_2H_5.	Decompose with hydriodic acid and estimate C_2H_5I (Zeisel procedure).	See *Methoxyl*, etc., pp. 116–118.
Ethoxyl.	O—C_2H_5.	(i) As for O—CH_3. (ii) Oxidise with chromic acid to acetic acid and distil this.	Kuhn and Roth, *Ber.*, 1933, **66**, 1274. See pp. 111–117.
Hydroxyl.	—OH.	See alcohol and phenol. Estimate "reactive hydrogen" by reaction with methyl magnesium iodide.	See pp. 118–121.
Ketone.	R—CO—R′.	See carbonyl for general reaction. Special colorimetric reactions: Acetone. Pyruvic acid.	See p. 320–321. See p. 325.
Methyl.	(C)—CH_3.	See alkyl.	
Methylimino.	N—CH_3 (also S—CH_3).	Decompose with hydriodic acid distilling several times; estimate iodide in methyl iodide formed.	See pp. 116–118.
Methylene-Ketone.	—CO—CH_2—	Colour reaction with dinitrobenzene and alkali.	See Part IV, p. 342.
Methoxyl.	—O—CH_3.	Decompose with hydriodic acid, collect methyl iodide, and estimate iodide.	See pp. 113–116.
Nitro (or Nitroso).	NO_2 (NO).	(i) Reduce quantitatively with titanous or stannous chlorides and back titrate excess. (ii) Reduce to aromatic amine, and estimate colorimetrically. (iii) Polarographic reduction.	See *Amino, primary aromatic*. Part VI, see p. 460.
Nitrate ester.	—O—NO_2.	(Nitrocellulose, etc.) (i) Reduce to nitric oxide with sulphuric acid and mercury and measure gas. (ii) Hydrolyse with alkali and estimate nitrate colorimetrically. (iii) Reduce to ammonia with Devarda's alloy.	See p. 319.
Olefine.	C=C.	See unsaturation.	

TABLE OF MICRO-CHEMICAL METHODS FOR THE ESTIMATION OF ORGANIC GROUPS—Continued

Group	Structural Formula	Method of Analysis	References
Phenol.	Aryl-OH.	(i) Acetylate or benzoylate and estimate acyl group.	See *Acetyl group.*
		(ii) Brominate with bromide-bromate and back titrate excess of bromine (for phenols of known structure).	Compare Part III, pp. 175–176.
		(iii) Couple with diazotised sulphanilic acid or *p*-nitraniline and estimate colorimetrically.	See p. 339.
		(iv) Estimate colorimetrically with Folin's phenol reagent.	See p. 339.
		(v) Estimate colorimetrically by the indophenol reaction.	See p. 340.
		(vi) Estimate "reactive hydrogen."	See p. 118.
Reactive hydrogen.	R—O—H $R''N$—H (sometimes CH_2—CO—, etc.).	React with methyl magnesium iodide and collect hydrogen.	See section D above, pp. 118–121.
Thiol.	—SH.	(i) Oxidise with iodine and back titrate excess.	
		(ii) For colorimetric procedures compare the estimation of *cysteine*.	See p. 352.
(Unsaturation.)	C=C. C≡C.	(i) Reduce catalytically and measure hydrogen uptake. Reduce catalytically and measure using Warburg's apparatus.	Part VI, pp. 568–570.
		(ii) React with bromine, Wij's solution, etc., and back titrate excess.	
		(iii) Gaseous unsaturated products are absorbed by fuming sulphuric acid.	Compare pp. 510–529.
Urea.		(i) Hydrolyse and estimate ammonia (various methods available).	Part III, pp. 161, 199.
		(ii) Decompose with nitrous acid and measure nitrogen.	
		(iii) Decompose with hypobromite and measure nitrogen. Methods (ii) and (iii) need empirical correction factors.	
		(iv) Special colorimetric reactions.	See pp. 345–346.
		(v) Precipitate with xanthydrol and weigh.	Fosse, *Ann. Chim.,* 1916, **6**, 13.

PART III

VOLUMETRIC ANALYSIS

VOLUMETRIC ANALYSIS

GENERAL PRINCIPLES

ACCURACY in volumetric analysis can be obtained only by making use of quantitative, rapid, chemical reactions which involve clearly distinguishable colour changes. Since the sensitivity of all volumetric methods is limited by the ease of visual perception, it is essential for micro-analysts to pay great attention to the choice of the optimum experimental technique.

Even with chemical reactions which themselves involve colour changes, e.g. titrations with potassium permanganate, it is impossible to dilute the reagents indefinitely with the object of improving accuracy by using larger, and thus more accurately measurable, volumes of liquid. Whilst the detectability of colour change can be brought to a much higher order of sensitivity by use of special colorimetric techniques (which are dealt with in Part IV of this volume), the usual limit of detectability of volumetric end-points is of the order of 0.01–1.0 μg. of reactant per ml. of solution. Readers should consult Feigl's monograph on "Spot Tests"* for tabulated data on the sensitivity of particular reactions.

The sensitivity of a volumetric process depends ultimately upon the *concentration of the reactants in the titration vessel.* Hence when fractions of a microgram of material have to be detected, micro-volumetric apparatus (pp. 142–157) must be used.

A titration is theoretically complete when a sufficiency of the titrating solution has been added to satisfy the stoichiometrical relationship between the reactants that can be expressed by a chemical equation, but inevitably a slight excess of the titrient must be added to obtain a visual colour change. The difference between this *visual end-point* and the theoretical end-point is called the *titration error.* In micro-titration it is essential that this error should be reduced to the absolute minimum.

With the majority of titration analyses, the titration error is dependent not so much upon visual perceptibility as upon chemical errors, arising from the fact that the reaction process is not, in extreme dilution (i.e. at about the theoretical end-point), an instantaneous, irreversible, chemical change unaffected by the other components of the titration mixture.

Chemical limits of accuracy in volumetric analysis are discussed below. They depend, naturally, upon the type of titration which is being performed, but since reaction velocities and degrees of dissociation and hydrolysis are

* Feigl, F., "Qualitative Analysis by Spot Tests" (translation), 1939; (revised edition, Elsevier Pub. Co., Amsterdam and New York, 1947).

functions of concentration, the aim of the analyst should be always to carry out his titrations in solutions of high concentration. For the main types of titration processes the following sections set forth essential physico-chemical principles, in the light of which the reader should be able to examine critically his own titration problems.

It is vital that the analyst should develop a sense of proportion about his results. It is useless to express a result in "significant figures" which are more precise than the experimental errors of the method, and there is obviously no point in using specially exact volumetric apparatus for a reaction in which the chemical error cannot be reduced to below a definite limit.

On the other hand, manipulative faults are the most likely sources of error, and a laboratory technique of the highest precision should be aimed at and rigorously maintained.

Theory of Acid-Alkali Titration

Indicators. The neutralisation of an acid by an alkali, and its converse, is essentially the combination of hydrogen and hydroxyl ions. This is the reversible process:

$$H^+ + OH^- \rightleftharpoons H_2O$$

for which the ionic product, K_w, is 10^{-14} at $22°$ C.

Both the hydrogen and the hydroxyl ion concentrations of *aqueous* solutions are commonly measured in terms of the expression pH, which is defined as the negative logarithm, to base 10, of the hydrogen ion *activity*. Thus an exactly neutral solution, containing 10^{-7} gram-equivalents of both H^+ and OH^- ions per litre, has a $pH=7$ (at $22°$ C.).

Acid-alkali titration depends, in principle, upon neutralising a solution to a constant pH, but, except in electrometric analysis (Part V) the practical attainment of an "end-point" involves the *recognition of a definite pH change*. For this purpose "indicators" are added to the titrated solutions. These are organic dyestuffs of weak acid or weak base character in which the colour alters over a definite pH range, due to tautomeric change consequent upon ionic dissociation or hydrolysis.

Since the production of a colour change, then, involves the neutralisation of the reactant in the flask *plus* approximately half the indicator added, the two essentials for accuracy are (i) to choose an indicator with as abrupt a colour change as possible, and (ii) to keep down to the lowest limits the amount of indicator used.

Table I gives a list of indicators in regular use.

TABLE I

INDICATORS FOR ACID-ALKALI TITRATION*

Indicator	Chemical Name	pH Range	Method of Preparation
Picric acid.	2:4:6-Trinitrophenol.	Colourless 0·0– 1·3 yellow.	0·1% in distilled water.
Thymol blue (acid range).	Thymol sulphone-phthalein.	Red 1·2–2·8 yellow.	Dissolve 0·1 g. in 10·75 ml. of $N/50$ NaOH and dilute with water to 250 ml.
Tropaeolin 00.	Sodium p-diphenyl-amine-azobenzene-m-sulphonate.	Red 1·3–3·0 yellow.	0·1% in water.
Methyl violet.		Blue 1·5–3·2 violet.	0·25% in water.
2 : 4-Dinitro-phenol.		Colourless 2·6– 4·0 yellow.	Dissolve 0·1 g. in a few ml. of alcohol and dilute to 100 ml. with water.
Methyl yellow.	Dimethylamino-azobenzene.	Red 2·9–4·0 yellow.	0·1% in 90% alcohol.
Bromophenol-blue.	Tetrabromophenol-sulphone-phthalein.	Yellow 3·0– 4·6 blue.	Dissolve 0·1 g. in 7·45 ml. of $N/50$ NaOH and dilute with water to 250 ml.
Congo red.	Sodium tetrazo-diphenyl-binaph-thoate.	Blue 3·0–5·2 red.	0·1% in water: filter carefully.
Methyl orange.	Sodium p-dimethyl-aminoazobenzene-sulphonate.	Red 3·0–4·4 yellow.	0·1% in water.
Sodium alizarine sulphonate (acid range).		Yellow 3·7– 5·2 violet.	0·1% in water: filter.
Bromocresol green.	Tetrabromo-m-cresol sulphone-phthalein.	Yellow 3·8– 5·4 blue.	Dissolve 0·1 g. in 7·15 ml. of $N/50$ NaOH and dilute with water to 250 ml.
2 : 5-Dinitro-phenol.		Colourless 4·0– 5·8 yellow.	Dissolve 0·1 g. in 20 ml. of alcohol and dilute to 100 ml. with water.
Methyl red.	Dimethylamino-azobenzene-o-carboxylic acid.	Red 4·6–6·2 yellow.	0·1% in alcohol.
Litmus.	Azolitmin.	Red 4·5–8·3 blue.	Grind 0·5 g. in 100 ml. of water and filter.
Cochineal.		Red 4·8–6·2 violet.	Grind 1 g. with 150 ml. of 50% alcohol, let stand for 2 days, and then filter.

* For further details see H. T. S. Britton, "Hydrogen Ions" (Churchill, London).

TABLE I

INDICATORS FOR ACID-ALKALI TITRATION†—*Continued*

Indicator	Chemical Name	pH Range	Method of Preparation
p-Nitrophenol.		Colourless 5·0–7·0 yellow.	0·1% in water.
Bromocresol purple.	Dibromo-o-cresol sulphone-phthalein.	Yellow 5·2–6·8 purple.	Dissolve 0·1 g. in 9·25 ml. of N/50 NaOH and dilute to 250 ml. with water.
Bromothymol blue.	Dibromothymol-sulphone-phthalein.	Yellow 6·0–7·6 blue.	Dissolve 0·1 g. in 8 ml. of N/50 NaOH and dilute to 250 ml. with water.
Neutral red.	Dimethyldiamino-toluphenazine.	Red 6·8–8·0 yellow.	Dissolve 0·1 g. in 70 ml. of alcohol, filter, and dilute to 100 ml. with water.
Phenol red.	Phenol sulphono-phthalein.	Yellow 6·8–8·4 red.	Dissolve 0·1 g. in 14·20 ml. of N/50 NaOH and dilute to 250 ml. with water.
Thymol blue (alkaline range).		Yellow 8·0–9·6 blue.	(See above.)
Phenolphthalein.		Colourless 8·2–10·0 pink.	1% in 60% alcohol.
Thymol-phthalein.		Colourless 9·3–10·5 blue.	0·1% in alcohol.
Sodium alizarin sulphonate (alkaline range).		Brownish-red 10·2–12·0 yellow.	0·1% in water.

For any indicator, as for any weak acid, $HIn \rightleftharpoons H^+ + (In^{*-})$ whence, by defining $K_{HIn} = \dfrac{[H^+] \cdot [In^{*-}]}{[HIn]}$ and $pK_{HIn} = -\log_{10}K_{HIn}$ it follows that

$$\frac{d[In^*]}{d[H^+]} = \frac{-C \cdot K_{HIn}}{([H^+] + K_{HIn})^2} \quad \dots\dots\dots\dots\dots\dots (A)$$

where C is the total indicator concentration, and

$$pH = pK_{HIn} + \log\frac{[In^*]}{[HIn]} \quad \dots\dots\dots\dots\dots\dots (B)$$

From equation (A) it follows that the smaller the amount of indicator added the more abrupt will be the colour change.

Equation (B) may be written:

$$pH = pK_{HIn} + \log\frac{\text{Colour of alkaline form}}{\text{Colour of acid form}}$$

† For further details see H. T. S. Britton, "Hydrogen Ions" (Churchill, London).

For most indicators the colour change is perceptible when about 25% of the indicator has been changed from the acid form to the alkaline form, or *vice versa*. Thus the transition range of an indicator may be reckoned, for direct inspection, as an interval of about a unit of pH, given by the equation

$$p\text{H}=p\text{K}_{\text{HIn}}\pm0.5.$$

The colour change is, however, much more sensitive with a one-colour indicator, such as phenolphthalein, than with a two-colour indicator such as methyl red. "Dichromatic" indicators, in which the apparent colour differs with the concentration or thickness of the solution, are still less sensitive unless used in measured constant amounts. The sulphone-phthalein dyes, such as bromocresol purple, belong to this category.

The abruptness of the colour change in acid-alkali titration may be increased greatly by the use of **" screened indicators,"** which at the transition point become colourless or grey. The "screening" of indicators may be effected by adding to the indicator solution another dye which has a colour exactly complementary to the "neutral" tinge of the indicator, thereby producing an "achromatic" mixture. For instance, if methylene blue is added to the indicator methyl red, the colour changes from violet, through the sensitive grey, to green as the solution is made more alkaline, and if the relative concentrations of the two colouring matters are adjusted properly, a pH change of 0.1 unit can be detected with ease. This corresponds to the neutralisation of only 10% of the indicator, and consequently by screening an indicator one can use an increased concentration without diminishing the sensitivity of the main titration. One is, in fact, making use of the principle of observing colour changes in monochromatic light (compare Part IV, pp. 224–227).

A number of sensitive " screened " indicator mixtures are listed in Table II below.

TABLE II

SCREENED INDICATORS

Composition	Transition Point	Acid	Alkaline
3 parts of 0.1% alcoholic bromocresol green and 1 part of 0.2% alcoholic methyl red.	5.1	Wine-red	Green
Equal parts of 1% aqueous methylene blue and 0.2% methyl red.	6.0	Violet	Green
Equal parts of a 0.1% aqueous solution of the sodium salt of bromocresol purple and of the sodium salt of bromothymol blue.	6.7	Yellow	Violet-blue
Equal parts of a 0.1% alcoholic solution of neutral red and methylene blue.	7.0	Violet-blue	Green
Equal parts of 0.1% aqueous solution of the sodium salts of bromothymol blue and of phenol red.	7.5	Yellow	Violet

Fluorescent indicators are occasionally of particular value. They may be used in even highly coloured or turbid solutions, e.g. with food extracts or with soil or clay suspensions. Suitable substances are listed in Table III.

TABLE III

FLUORESCENT INDICATORS

Indicator	Colour Change	pH Range
Benzoflavine	Yellow to green	0·3–1·7
Eosin	Colourless to green	2·5–4·5
Eosin + Xylene Cyanol FF	Colourless to green	4·0–4·5
Dichloro-fluorescein	Colourless to green	4·0–6·0
Fluorescein	Colourless to green	4·0–4·5
Erythrosin	Colourless to green	4·0–4·5
Chromotropic acid	Colourless to blue	3·5–4·5
Resorufin	Yellow to orange	4·4–6·4
Acridine	Green to violet	4·9–5·1
β-Methyl umbelliferone	Colourless to blue	6·5–7·5
Umbelliferone	Colourless to blue	6·5–7·6
Quinine	(a) Blue to violet (b) Violet to colourless	5·9–6·1 9·5–10·0
α-Naphthol	Colourless to blue	6·0–8·0
α–Naphthol sulphonic acid	Dark blue to violet	9·0–10·0

Buffered Solutions. The general equation $pH = pK_a + \log \frac{[\text{Anion}]}{[\text{Acid}]}$, which was developed on p. 133 with reference to indicators, holds for the neutralisation of any weak acid. Taking into account the possibility of aqueous hydrolysis, this may be written

$$pH = pK_a + \log \frac{\text{Unhydrolysed salt form}}{\text{Undissociated acid form}},$$

whilst for the neutralisation of a weak base the corresponding equation is

$$pH = pK_b - \log \frac{\text{Unhydrolysed salt form}}{\text{Undissociated base form}}.$$

Thus during the neutralisation of either a weak acid or a weak base the pH changes but slowly as the titration proceeds. Partially neutralised solutions of this type are said to be "buffered."

In the region of a stoichiometric end-point $d(p\mathrm{H})/d$(reagent) changes rapidly, thus:

for 99% neutralisation of a weak acid we must have $p\mathrm{H}=p\mathrm{K}_a+2$,

for 99·9% neutralisation of a weak acid we must have $p\mathrm{H}=p\mathrm{K}_a+3$,

and consequently *for the accurate titration of a single acid or base one should choose an indicator having* $p\mathrm{K}_{\mathrm{HIn}}$ *differing by at least two units from the* $p\mathrm{K}$ *of the main reactant.*

Thus for titrating weak acids a strong base and an indicator of high $p\mathrm{K}_{\mathrm{HIn}}$ with a colour change in the region of $p\mathrm{H}$ 8–10, such as phenolphthalein should be used; a weak base, such as ammonia, should be titrated with a mineral acid and an indicator of low $p\mathrm{K}_{\mathrm{HIn}}$, such as methyl red, with a colour change in the region of $p\mathrm{H}$ 4–6.

A strong acid may be titrated in the presence of a weak acid by using an indicator with a colour change at $p\mathrm{H}$ 2–4, such as 2 : 4-dinitrophenol or thymol blue, and a strong base may be titrated in the presence of a weak base by using an indicator with a colour change at $p\mathrm{H}$ 10–12, such as sodium alizarin sulphonate or thymol-phthalein. Illustrative examples of these types of titrations are given on pp. 161–166.

When one has to deal with a mixture of weak acids (or bases) or with polybasic weak acids, such as citric acid, it may be impossible to find conditions such that $d(p\mathrm{H})/d$(reagent) changes rapidly. In this case it is necessary to titrate to a fixed $p\mathrm{H}$ (i.e. to a pre-determined colour; see p. 166 for an example) and work under carefully standardised conditions. Electrometric titration (Part V, pp. 410–433) should be resorted to if a high degree of sensitivity is required.

Titration Errors due to Carbon Dioxide. In micro-chemical titrations the attainment of accurate end-points may often be hindered by the presence of dissolved carbon dioxide, since carbonic acid ($p\mathrm{K}_{a1}=6{\cdot}5$; $p\mathrm{K}_{a2}=10{\cdot}2$) can act as a buffer. For this reason baryta is a much safer reagent to use than caustic soda: micro-burettes and storage flasks for the latter reagent should always be protected by soda-lime guard tubes. Alkaline solutions before titration should always be stored in stoppered vessels.

Even acid solutions may, by exposure to air, dissolve carbon dioxide until the solution contains as much as $10^{-5}N$ carbonic acid. 100 ml. of such a solution would neutralise 0·1 ml. of $N/100$ alkali—quite a significant titration error. To avoid this error the volume of liquid in the titration flask should be kept to the minimum and the solution should be boiled for a few seconds and then cooled immediately before titrating. Excessive dilution of a reactant with water from a wash-bottle is to be avoided, since "distilled water" soon becomes charged with carbon dioxide; which, however, can easily be removed by boiling the water in a vessel of hard glass.

Solvent Errors. The published values of indicator constants, $p\mathrm{K}_{\mathrm{HIn}}$, refer only to their *cold, dilute* solutions *in distilled water*. Neutral salts

in high concentration can introduce errors by altering ionic strengths and repressing the ionisation of weak electrolytes.

Grave errors can result both from the use of warm solutions and from the presence of any appreciable percentage of another solvent, such as alcohol. The term pH is meaningless in non-aqueous solutions; moreover, the dissociation constants of different acids and bases do not respond uniformly to solvent changes. If non-aqueous solutions have to be titrated, then a comparison tube (pp. 158–159) should always be used.

Theory of Oxidation-Reduction Titrations

Oxidation-reduction reactions involve electron transfer from the oxidised substance to the reduced substance, e.g.:

$$Fe^{++} - e \rightleftharpoons Fe^{+++}.$$

For all *reversible* processes the course of the chemical change can be followed electrically (see Part V) and the **oxidation potential** E_h, of any solution, measured as the potential difference in volts between (a) an inert electrode (e.g. a clean platinum wire), and (b) a normal hydrogen electrode inserted into the liquid, gives a measure of its capability to oxidise or reduce. A strong oxidiser will tend to remove electrons from the inert electrode, with the result that it will attain a positive electrical potential; in strong reducing agents the electrode acquires a negative potential.

In accordance with electro-chemical theory, one can write for the simple process *Oxidiser^0 + n . e $\rightleftharpoons$ Reducer^{-n}*:

$$E_h = E_{o'} + 2 \cdot 3 \frac{RT}{nF} . \log_{10} \frac{[\text{Oxidiser}]}{[\text{Reducer}]} = E_{o'} + \frac{0 \cdot 058}{n} \log_{10} \frac{[\text{Oxidiser}]}{[\text{Reducer}]}$$

where $E_{o'}$ is the value of E_h for the half-oxidised system under the given reaction conditions.

Thus for the change between 0·1% and 99·9% reduction of a compound, the oxidation potential of the solution changes by only 0·35 volt for a single electron transference (e.g. $Fe^{+++} \rightleftharpoons Fe^{++}$) and by $1/n$ of this value for a n electron transference.

Consequently, if two reagents, A and B, react:

$$A_{Oxidised} + B_{Reduced} \rightleftharpoons A_{Reduced} + B_{Oxidised}$$
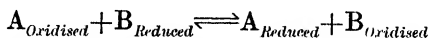
then the chemical change will take place completely if the oxidation potential $E_{o'}$ of B is 0·35 volt lower than that of A. Unless there is this potential difference between the two reactants then an oxidation-reduction titration will not reach a stoichiometric end-point, but an equilibrium value.

Moreover, if an oxidiser reacts with a solution containing two reducing agents, B and C, then C will be oxidised completely before B is attacked if the oxidation potential of C is more than 0·35 volt below that of B.

Under these conditions the commencement of oxidation of B can be taken as a sign of complete oxidation of C. This is the underlying principle in the use of **oxidation-reduction indicators,** which are, in practice, organic dyestuffs which have highly coloured, quinonoid, oxidised forms and practically colourless reduced forms, or else complex co-ordination compounds of metals, such as iron, with two valency states. Table IV lists oxidation-reduction indicators in common use.

TABLE IV

OXIDATION-REDUCTION INDICATORS

Indicator	Colour Change	Indicator for
Diphenylamine	Colourless to violet	Dichromate titrations (see p. 172)
Diphenylamine-sulphonic acid or barium salt	Violet to green	Ferrous iron in presence of tungstate
Diphenyl-benzidine or its sulphonate	Colourless to violet	Dichromate titrations (see p. 172)
Diphenylcarbazide	Pink-red to green	Dichromate titration of iron
Erioglaucin A	Green to pale grey	Permanganate titration of ferrocyanides and ferrous iron
Methyl-orange	Yellow to colourless	Bromate titrations (see p. 175)
o-Phenanthroline		Ceric sulphate, etc., titrations of ferrous iron (see p. 170)
N-Phenylanthranilic acid	Crimson to violet	Vanadium, chromium, iron
Xylene cyanol FF	Green to orange	Ceric sulphate titrations
Dianisidine	Colourless to purple	Ferro and ferricyanides (e.g. estimation of zinc, see p. 186)

The practical precautions to be taken in the use of oxidation-reduction indicators are very similar to those needed in the use of acid-alkali indicators.

The term $E_{o'}$ of the preceding equations is dependent upon the hydrogen ion concentration of the solution which contains the oxidising or reducing agent, since though the potential acquired by the inert electrode depends only upon the oxidation-reduction process, the potential difference between the reference hydrogen electrode and the solution depends upon its pH. A solution of acidity $[H^+]$ in contact with a hydrogen electrode is at potential $RT/F \times \log e[H^+]$, or $-0.058\ p$H. Consequently $E_{o'} = E_o - 0.058\ p$H and

$$E_h = E_o + \frac{0.058}{n} \log_{10} \frac{[Ox.]}{[Red.]} - 0.058\ p\text{H}$$ where E_o is characteristic only of

the dissolved reagent and is termed the *standard oxidation-reduction potential* of the reagent.

From the above equation it will be seen that the oxidation potential of a solution decreases as its acidity decreases. In other words, acid conditions favour oxidation and alkaline conditions reduction.

Consequently *oxidation-reduction indicators will only give consistent results if they are used under conditions of constant pH.* This point is of particular importance in the titration of biological systems, which should always be analysed in buffered solutions. For this reason, too, reagents such as iodine and sodium arsenite do not give correct results unless they are used in solutions of controlled acidity.

The electro-chemical equations given above are not valid for reactions irreversible under the experimental conditions (e.g. $MnO_4^- \rightarrow Mn^{++}$ or $RCHO \rightarrow RCO_2H$), though the approximate potential change can often be estimated from the heat of reaction. However, even irreversible reactions appear to set in at quite definite oxidation potentials, so that in these cases, too, oxidation-reduction indicators can often be used with advantage. The irreversible oxidation of an indicating dyestuff is, however, a practical difficulty that may be encountered (e.g. when using diphenylamine). Whilst oxidation-reduction indicators are usually stable in reducing solutions they should never be allowed contact for any time with excess of strong oxidisers, such as potassium dichromate or ceric sulphate. Consequently the end-point of an oxidation titration should be approached slowly and the solution kept thoroughly stirred.

Many of the oxidation-reduction processes which involve irreversible chemical changes are comparatively slow reactions, and unless special care is taken to anticipate this difficulty large and variable titration errors may result.

One helpful procedure is to add a third substance which by acting as a *potential mediator* sets up immediately in the solution a reversible oxidation-reduction system involving one of the main reagents. Thus manganous sulphate can be added to accelerate permanganate titrations (p. 167), whilst a ferric salt may be added when titrating ferrous salts with dichromate using diphenylamine as the indicator, to set up immediately the reversible system $Fe^{++} \rightleftharpoons Fe^{+++} + e$ and to convert the diphenylamine to the more complex dyestuff which is the true indicator.

Alternatively, an excess of the oxidiser or reducer may be added and after a brief interval back titrated with a third rapidly reacting substance. Thus iodine is an oxidiser which should always be added in excess and then back titrated with sodium thiosulphate or sodium arsenite. A further advantage in this case is that losses due to volatilisation are minimised, whilst again the abrupt disappearance of the blue starch-iodine colour is an ideal end-point.

Side reactions are often sources of error in oxidation-reduction titrations, particularly when only one component of a complex mixture is being estimated. A preliminary purification process is often necessary, but

sometimes the difficulty may be overcome by the addition of reagents which form complexes with the interfering substance or precipitate it from solution.

Theory of Precipitation Titrations

The sensitivity of the end-point in precipitation analysis depends mainly upon the solubility product of the precipitate; the lower the solubility of the precipitate the more abrupt the critical ionic concentration change in the solution, and consequently the sharper the colour change due to the indicator and the more dilute the reagent solutions which can be used.

Two types of indicators are in common use in precipitation analysis: (a) reagents which give intense colours when one of the reactant ions is present in excess (e.g. ferric salts for detecting the thiocyanate anion), and (b) adsorption indicators, i.e. dyestuffs which selectively adsorb on the precipitate according to the resultant electrical charge on its particles. Indicators of type (a) are usually less sensitive than those of type (b) on account of the greater tinctorial power of dyes, but in general, type (a) indicators can be used with accuracy over a wider range of experimental conditions.

Adsorption indicators act by depositing, from solution, on to the surface of the precipitate when its net electrical charge changes sign. Thus in titrating a chloride with silver nitrate using phenosafranine or fluorescein, the precipitate AgCl has a net negative charge so long as chloride ions are present in excess in the solution, since the minute silver chloride crystals have some chloride ions adsorbed on their surfaces. As the stoichiometric end-point is passed the AgCl particles gain a net positive charge, since they then have silver ions in excess. Thereupon the dyestuffs, which are anions, adsorb on to the precipitate, producing an abrupt colour change.

Table V lists a number of commonly used adsorption indicators.

TABLE V

COMMON ADSORPTION INDICATORS

Indicator	Colour Change	Indicator for
Alizarin	Red to grey-green	Nitrates
Diiodo-fluorescein	Yellow to pink	Chlorides, iodides
Diphenyl-carbazide	Pink to violet	Alkali cyanides
Fluorescein (soluble)	Green fluorescence to pink	Chlorides, bromides, iodides
Phenosafranine	Pink to blue	Chlorides, bromides
Tetraiodo-fluorescein (erythrosine R)	Pink to bluish-pink	Iodides
Tetrahydroxyquinone	Yellow to pink	Sulphate

These indicators are generally used in 0.1% solution in water or alcohol.

Though, in general, inorganic anions (NO_3^-, SO_4^{--}, etc.) are much less readily adsorbed on to positively charged precipitates than are the anions of dyestuffs, they may be, when present in high concentration, adsorbed sufficiently to inhibit the prompt colour change. "Salt errors" must therefore be guarded against in precipitation analysis, particularly when easily adsorbed ions are present in the solution. Again, since the majority of adsorption indicators are dyestuffs capable both of dissociation and hydrolysis, their behaviour may vary with change of pH of the solution. Thus the titration of organic salts (e.g. the determination of the equivalent of a quaternary ammonium halide) often presents difficulties.

The failure of adsorption indicators is still more evident when the solution contains protective colloids, such as proteins. Consequently adsorption indicators are of little value in biochemical work.

Indicators of soluble ions are much less prone to be affected by "salt errors," though specific interactions between reagents to form complex anions (e.g. combination of ferric salts with fluorides or oxalates) should be guarded against.

Organic compounds which form coloured co-ordination complexes with metals are now proving to be particularly valuable as indicators in precipitation analysis. Thus the micro-chemical method of estimating fluorides (p. 191) depends on the production of an intensely coloured thorium "lake" when formation of the less soluble thorium fluoride is complete.

Many further volumetric processes of this type can be developed on the basis of characteristic "spot tests" for metallic ions. In this connection one may instance the use of mercuric nitrate for the estimation of chloride ions (p. 182). Mercuric chloride, though soluble in water, is undissociated in nitric acid of pH 2 or less, while mercuric nitrate ionises even in strong acid. Consequently diphenyl-carbazide, which gives a purple mercury complex, can be used as a very sensitive indicator for titrating a chloride solution with mercuric nitrate, provided that the pH of the solution has been properly controlled by the addition of nitric acid.

MICRO-VOLUMETRIC APPARATUS

DURING the course of development of micro-volumetric methods of analysis, for which biochemists have of necessity been primarily responsible, it soon became evident that the standard forms of macro apparatus were often quite unsuited to deal with liquid volumes of which the upper limit was of the order of 5 ml. Numerous examples of micro-volumetric pipettes and burettes have been described in chemical literature,* and many of these are to be found in catalogues issued by manufacturers of scientific glassware, but volumetric flasks do not seem to have received the same attention.

The discussion of micro-volumetric apparatus which follows should help to guide the micro-analyst in choosing suitable apparatus from a bewildering variety of designs, and in acquiring the requisite laboratory technique.
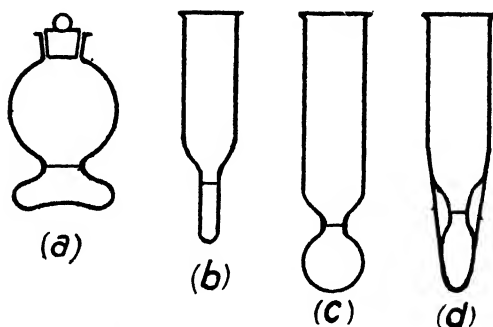
Fig. III.1. Volumetric Flasks.

Volumetric Flasks. The standard form of volumetric flask is satisfactory down to a capacity of 5 ml. if the graduation is near the bulb. It is of no value in smaller sizes, since adequate mixing becomes impossible and losses due to trapping by or leakage past the stopper assume serious proportions. These objections are overcome by the designs shown in fig. III.1; (a) is due to Caley,† while (b), (c), and (d) are recommended by Holmes.‡

In all cases mixing is effected in the large upper chamber. These flasks are very convenient for multiple operations, e.g. type (c) can be used for a wet digestion, the digest subsequently being made up to volume, while type (d) is developed from a centrifuge tube. It is particularly useful for concentrating micro-precipitates from large volumes of solution.

* A specialised review with extensive bibliography is given by Wyatt, *Analyst*, 1944, **69**, 81.
† Caley, E. R., *Ind. Eng. Chem.* (Anal. Edn.), 1941, **13**, 204.
‡ Holmes, F. E., ibid., p. 586.

Pipettes. Micro-pipettes may be divided into three main groups:

(a) "Wash-out" pipettes, i.e. those calibrated to contain a given volume.

(b) Delivery pipettes, i.e. those calibrated to deliver a given volume.

(c) Dilution pipettes.

It is not possible to state categorically where each pattern should be used, since a number of factors are involved; but every micro-analyst should have a selection from which to choose the most suitable for a given purpose. Some guidance on this point is given in the table at the end of the section, and it should be noted that many of the pipettes described are best made in the laboratory.

"*Wash-out*" *Pipettes.* The many patterns described in the literature are mostly similar in design, variations being mainly concerned with filling. Pregl and Wortall* describe "precision wash-out" pipettes of about 0·1 ml. capacity. These are a variation of the well-known Ostwald type (fig. III.2, *a*), but a straight type (fig. III.2, *b*) is generally to be preferred. Such pipettes are easy to make from narrow tubing of bore such that the graduation lies between 5 and 10 cm. from the tip.

If held in an inclined position, this type of pipette will usually fill by surface-tension effects alone.

Fig. III.2, *c* illustrates an elaboration of the straight pipette, designed by Wigglesworth† for the measurement of volumes of the order of 0·3 cu. mm.

The capillary *A* is 5–10 cm. long with a bore to suit the volume required, and the lower end is waxed inside and out.

Fig. III.2. Wash-out Pipettes.

If the waxed end is dipped below the surface of the solution until completely covered the liquid will rise to the top of the tube. The pipette is emptied by blowing down the rubber tube attached to the outer protecting tube *B*.

When filling a wash-out pipette (which should be dry initially), the meniscus should not be allowed to pass the line by more than 1 mm. After wiping the outside of the tip, the meniscus may be brought exactly to the mark by judicious application of a fragment of filter-paper. The pipette should be washed and dried before it is used again. This can often be done
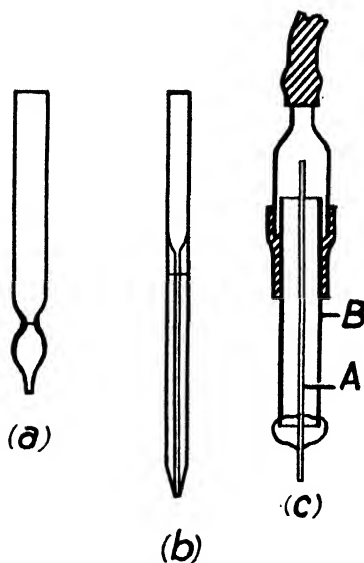
* Pregl, F., "Quantitative Micro-analysis," p. 117, London, 1930.
† Wigglesworth, V. B., *Biochem. J.*, 1927, **21**, 791; 1937, **31**, 1719.

in a few minutes by attaching the pipette to a filter-pump and sucking a few millilitres of acetone through it, followed by a current of air. The practice of rinsing out with the solution to be measured is strongly to be deprecated, since there is danger of leaving a film of liquid above the graduation; this on washing out is added to the main volume of the pipette.

Delivery Pipettes. These may be classified in three main groups:

(*a*) Those emptied by simple drainage under the influence of gravity.

(*b*) Those drained as in (*a*) and then blown through once to dislodge the drop of liquid remaining.

(*c*) Those emptied mechanically.

In class (*a*), the bulb pipette, familiar to every chemist, is obtainable down to 1 ml. capacity. It is not always appreciated, however, that these pipettes, if of small capacity, may be subject to errors as great as 5% when used for measuring liquids such as alcohol. If liquids differing markedly from water in such properties as surface tension and viscosity are to be measured, the pipette must be calibrated accordingly.

For amounts less than 2 ml. it is preferable to use a pipette delivering between two marks, since errors due to variations in the volume of retained liquid or to accidental damage to the tip are thereby eliminated. Straight graduated pipettes are very satisfactory and are obtainable in a wide range of sizes. Those of 1 ml. capacity are usually graduated to 0·01 ml., while quite accurate pipettes of 0·1 ml. total capacity, graduated to 0·002 ml., are obtainable.

Another type of pipette designed to deliver between two marks is that recommended by Van Slyke* (fig. III.3, *a*). The tap should be of good quality with the bore of the key free from chipping and in line with the capillary tubes. These pipettes are extremely accurate and most convenient to use. They deserve much wider application.

In all cases where liquid is delivered from a pipette under the influence of gravity, the rate of fall of the meniscus should not exceed 0·5 cm. per second. This allows sufficient time for drainage and enables the meniscus to be stopped exactly at the lower mark.

Blow-out Pipettes. These are generally similar to plain delivery pipettes, being used in the same manner except that the drop of liquid remaining is removed by blowing once through the pipette. They are not so accurate as the "wash-out" pipettes, but are of value when dealing with fairly viscous liquids or for measurements in which extreme economy of material is essential and the "wash-out" technique is not applicable.

Delivery Pipettes Emptied Mechanically. This type of pipette seems to be peculiar to the biochemical field, though its manifest advantages fit

* Peters and Van Slyke, "Quantitative Clinical Chemistry," 1932.

it for use in other spheres. It always incorporates the "syringe" principle, though the piston may be solid, liquid, or occasionally gas.

The type introduced by Krogh* (fig. III.3, b) is one of the most useful. It is made from an "all-glass" vaccine syringe common in medical practice. The syringe A is fitted with an ebonite collar B to which two steel rods C, carrying a bracket D and stop screw E, are attached. A fibre block F prevents rotation of the piston in relation to the barrel of the syringe. The arrangement of the steel rods also compensates for variations in the volume of the syringe due to changes in temperature.

In the original model, the syringe was used without modification, i.e. the jet consisted of a stainless-steel hypodermic needle. This has the disadvantage that air bubbles trapped at the junction of the needle and syringe cannot be seen, while the overall length of the jet is short. The glass capillary fused to the syringe, as depicted in the figure, is a great improvement and permits of the syringe being used for the measurement of very small volumes. Suitable syringes are available in 10, 5, 2, and 1 ml. capacities, the better qualities being manufactured to remarkably fine limits. By adjustment of the stop-screw, any volume less than about 75% of the nominal capacity of the syringe may be delivered with great accuracy. It is not desirable to use the full capacity, since the length of the piston remaining in the barrel is then rather short. These syringes are equally suitable for liquids of widely varying

Fig. III.3. Delivery Pipettes.
(a) Van Slyke pattern. (b) Krogh pipette. (c) Trevan pipette.

properties and are invaluable for use with solutions which must not be exposed to the atmosphere. It is quite possible to deliver amounts of 10 ml. or less with an error of only 0·0001 ml., though the usual variation with the larger sizes is 0·0005 ml.

A minor disadvantage when dealing with quantities less than about 0·1 ml. is the relatively large "dead-space"; but if the volume required is less than that of the capillary, mercury may be introduced so that when the glass piston is at the bottom of its stroke, the capillary is completely
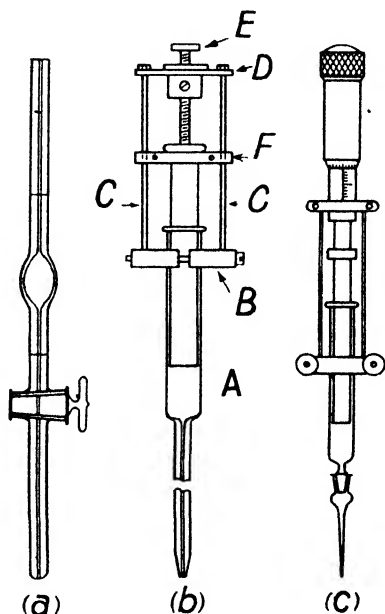
* Krogh, A., *Ind. Eng. Chem.* (Anal. Edn.), 1935, **7**, 130.

filled. The mercury then acts as the piston and the capillary as the barrel. No difficulty is experienced in washing out the Krogh syringe between filling with different solutions.

Benedetti-Pichler* utilises a similar principle for the delivery of ultra-micro quantities, but the actual amount is determined by observation under the microscope.

The Micrometer Syringe. This type of pipette (fig. III.3, *c*) was introduced by Trevan† and may also be used as a micro-burette. Like the Krogh pipette, it is based on a vaccine syringe, the piston being controlled by a micrometer screw. Such pipettes are available with a total capacity of 1 ml., and of such dimensions that each turn of the thimble delivers 0·02 ml., while the smallest division corresponds to 0·0002 ml. They are particularly valuable where it is necessary to deliver a series of closely-graded quantities, or for the production of uniform micro-drops of liquid.

Dilution Pipettes. Dilution pipettes are very useful when it is necessary to dilute a liquid in a definite ratio. This piece of apparatus seems to have escaped general notice, although it has been in use by haematologists for many years.

Fig. III.4 illustrates a so-called "red-cell" pipette. In effect it is a "wash-out" pipette *A* combined with a micro-volumetric flask *B*. The liquid to be diluted is drawn up to the mark *1*, the tip is wiped carefully and the diluting fluid is drawn in until the mark *101* is reached. The bulb contains a glass bead to assist in mixing, while the capillary is sufficiently fine to prevent diffusion. Before using the diluted liquid, the diluent remaining in the capillary must be expelled. If for any reason the full final volume of solution must be retained, a third mark should be arranged at *100* and when removing the excess diluent from the pipette (i.e. that contained between the tip and the mark *1*), the meniscus is stopped when it reaches this point so that no loss of solution occurs.

Pipettes of this type, for dilution in the ratios 1 : 100 or 1 : 10 and of about 1 ml. total capacity, are obtainable as "red-cell" and "white-cell" blood counting pipettes. Pipettes having other ratios can be made quite readily in the laboratory.

Micro-burettes. Very many different designs of micro-burettes are in common use, but since the technique of micro-titration requires more attention to detail than does its macro counterpart, the analyst should be thoroughly familiar with his equipment. It is advisable, therefore, to make

Fig. III.4.
Dilution Pipette.

* Benedetti-Pichler, A. A., *Ind. Eng. Chem.* (Anal. Edn.), 1937, **9**, 483.
† Trevan, J. W., *Biochem. J.*, 1925, **19**, 1111.

TABLE VI

SUMMARY OF MICRO-PIPETTES

Type	Manner of Delivery	Optimum Capacity	Application and Notes
"Pregl," etc. (p. 143).	Drain and wash out.	0·02–0·2 ml.	Suitable where contents can be delivered into a miscible liquid. Accuracy unaffected by viscosity, etc.
"Wigglesworth."	Blow and wash out.	0·02 ml. or less.	As above, but troublesome with viscous liquids.
Bulb (p. 144).	Drain out.	2 ml. and over.	Generally for aqueous solutions. Requires special calibration for other media. Not suitable for viscous liquids.
"Van Slyke" (p. 145).	Drain between two marks.	0·5–10 ml.	Calibration not affected by surface tension and can be used with moderately viscous liquids.
"Krogh" (p. 145).	Positive, glass syringe.	0·2–10 ml.	Calibration quite unaffected by nature of liquid. Capable of extreme precision and rapid in action. Can be adapted for smaller quantities if mercury no objection.
Micrometer syringe (p. 146).	Positive, glass syringe.	1 ml.	Similar to Krogh. Suitable for successive delivery of very small volumes.
Dilution pipette.	Wash-out pipette and integral "flask."	Pipette up to 0·1 ml., "flask" as required.	Very valuable for accurate dilution of small quantities of liquid.

a careful selection of apparatus and to keep to one or two types of burettes, as far as possible, in order that the manipulative technique shall become quite automatic.

There are two main groups into which all micro-burettes may be placed, viz.

(a) burettes emptied by gravity alone;

(b) burettes in which a "positive" method of delivery is used.

The earliest micro-burettes fall into the first category and were "scaled-down" models of the standard macro pattern. Such burettes are still available down to a capacity of 1 ml., graduated to 0·01 ml. They are filled by sucking the fluid into the burette from a vessel held under the tip. This procedure is far from satisfactory and led to Bang's* introduction of the integral reservoir (fig. III.5), a further modification being the provision of a much larger reservoir (e.g. a 500 ml. aspirator) connected at A by

* Bang, I., "Mikromethoden zur Blutuntersuchung," Munich, 1920.

rubber tubing. This pattern of burette is of the most value in the range 1–10 ml., providing, as mentioned in connection with pipettes, the rate of fall of the meniscus is not more than 0·5 cm. per second.

Eissner* introduced the "calibrated reservoir" burette shown in fig. III.6, enabling quantities of reagent greater than 5 ml. to be measured accurately: this type is limited in application.

In many micro procedures the volume of titrient is much less than 1 ml., and many special burettes have been devised to measure such quantities with an accuracy equal to that obtained in standard macro titrations. Further scaling down of the normal vertical patterns is impracticable
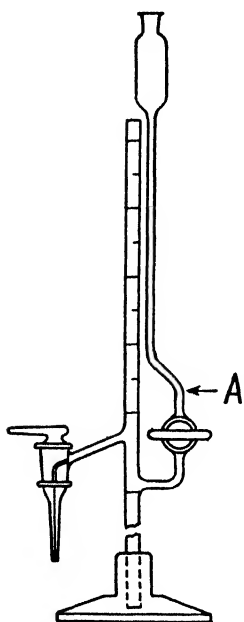
Fig. III.5. Bang Burette.   Fig. III.6. Eissner Burette.
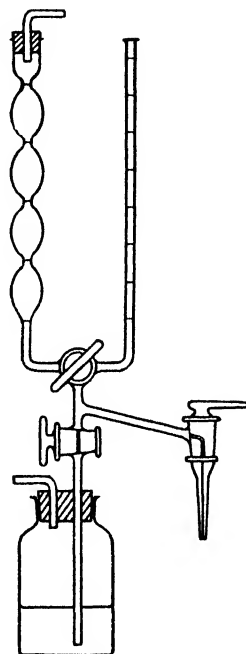
because of drainage errors. Since the separation of individual graduations on a burette should not be less than about 1 mm., it follows that a burette of 0·25 ml. capacity and 25 cm. long (1 cu. mm.=0·001 ml.) would have a bore of only 1·1 mm. Owing to the high ratio of surface area to volume, the delivery from such a burette, if of normal pattern, would need to be extremely slow to allow adequate time for drainage.

The horizontal burette of Conway† largely eliminates this difficulty. Originally, a capacity of 0·25 ml. was recommended, but the arrangement

* Eissner, W., *Zeit. Anal. Chem.*, 1932-3, **91**, 172.
† Conway, E. J., *Biochem. J.*, 1934, **28**, 283.

is useful in capacities between 1 ml. and 0·1 ml. It is particularly valuable for serial acid-alkali or oxidation-reduction titrations and where the reagent must be kept from contact with the air.

The main disadvantage of this and other similar burettes is the tendency of particles of grease to block the jet.

Fig. III.7 illustrates a slightly modified version of the Conway design. The assembly is mounted on a wooden stand by means of spring clips to facilitate stripping for cleaning or repair. The calibrated tube A is attached to the main part of the burette by a rubber joint at each end, thus allowing different-sized tubes to be substituted readily. To ensure that the reagent used in a titration has never been in contact with the rubber, the lower horizontal tube B is at least 2 mm. in diameter. Thus the volume contained between the **T**-joint and the rubber connection is considerably greater than
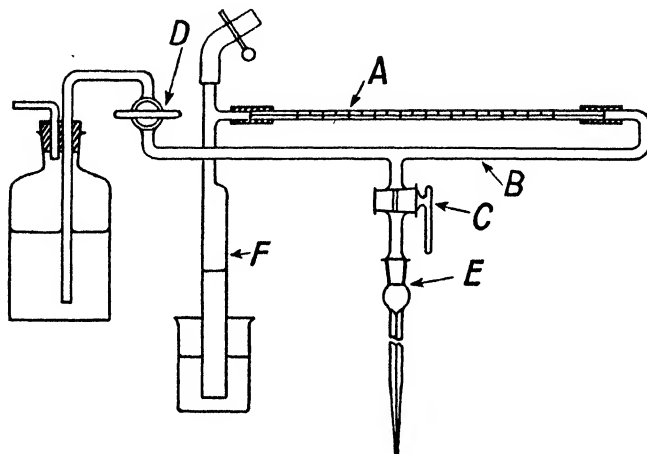
Fig. III.7.   Conway Horizontal Burette.

the volume of the larger calibrated tube. The control tap C is provided with a long handle to facilitate delivery of very small amounts. Jets are interchangeable at the ground joint E. The lower end of the vertical tube F dips into a vessel of water, while the top is closed by a rubber tube and Mohr clip. This vertical tube serves as a brake, the rate of travel of the meniscus along the calibrated tube being controlled by the height of the column of water. It should be at least 1 cm. wide, to minimise the change of level and, hence, of the rate of delivery during a titration.

The burette is best filled by closing the tap D and sucking water almost to the top of F. A beaker of reagent is placed under the jet and tap C opened, the solution being allowed to rise just to the level of the horizontal tube. Tap C is then closed, suction again applied to F and tap D opened to allow the solution from the stock bottle to fill the two horizontal tubes, D being closed when the meniscus reaches the zero mark on A.

In the original model the taps C and D were arranged with the bores horizontal when in the open position. The vertical disposition illustrated makes it much easier to remove any air bubbles trapped in the system.

Two simpler horizontal burettes due to Wilson are shown in fig. III.8, a and b. In (a) filling is achieved by applying pressure to the stock bottle and opening the lower tap until the liquid reaches the zero mark on the calibrated tube A. The end of the choke tube B is then closed with the finger and the top tap opened to fill the jet. When full, both taps are closed and the titration made by manipulating the upper one. Type (b) is first filled by suction applied to the reservoir, the reagent being drawn through the jet with both taps open and the calibrated tube sealed as above. When the liquid reaches the bottom of the reservoir both taps are closed and more
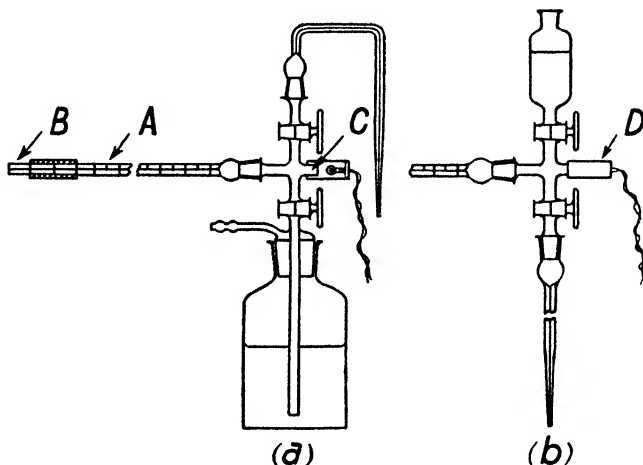
Fig. III.8.   Wilson's Horizontal Burette.

reagent can be added through the top. The upper tap is then opened to fill the calibrated tube, while in this case titration is carried out by means of the lower tap. In both types the calibrated tubes are interchangeable at the standard joint so that one tap unit may be used for a series of burettes of different volumes. The capillary choke B serves to control the rate of delivery. To facilitate the reading of the meniscus, a piece of glass rod C is fused to the burette as shown and carries a flash-lamp bulb in a small ebonite holder D.

Light from the bulb suffers total internal reflection along the column of liquid so that the meniscus appears brilliantly illuminated and may be adjusted with great ease. These burettes are readily constructed in the laboratory by anyone with moderate glass-blowing skill.

Gravity burettes in which the tap does not come in contact with the

reagent have been described by Benedetti-Pichler,* Pauli and Semmler,†
and others. Such burettes also incorporate a capillary brake to render
control more precise. They are, however, rather tedious to use, and if
taps must be avoided one of the positively operated types, considered in
the next section, is to be preferred.

"*Positive*" *Burettes.* For titrations of the order of 0·1 ml. "tapless,"
positively operated burettes are recommended. Such burettes usually
incorporate a screw device to control a mercury column, and either the
burette capillary or the screw may be calibrated.

The pattern best known is that of Rehberg‡ (fig. III.9). The body of
the burette *A* consists of a length of fine capillary tube of about 0·9 mm.
bore graduated in 2 mm. divisions, so that each division corresponds to
0·001 ml. approximately. The lower end of the capillary
is fused to a wider piece of tube *B* which forms a
mercury reservoir. A steel screw *C* is fixed into the
open end with sealing wax, the wax being, in effect, a
nut cast round the screw. The burette is provided with
a detachable jet *E* of greater volume than the body of
the burette so that the reagent used in the titration
does not come into direct contact with the mercury.

The Rehberg burette is fairly satisfactory and may
be read to 0·0001 ml., but in common with other
burettes of similar design (compare Pincussen§), leak-
age of mercury past the screw develops after some use.
It is, in fact, extremely difficult to make a screw and
nut by any method which does not leak under a
pressure of 10–20 cm. of mercury, but in burettes
of the Rehberg pattern the error so produced is not
so serious as in those where the screw is calibrated. On

Fig. III.9.
Rehberg Burette.

no account should grease be used in an attempt to stop
such leakage, since it readily emulsifies the mercury
and soon finds its way into the capillary, rendering the burette quite useless
until stripped and cleaned.

Widmark and Örskov‖ describe a vertical burette of 0·2 ml. capacity in
which the mercury column is advanced by an accurately fitted steel piston
controlled by a micrometer screw. While this system obviates leakage,
the large volume of mercury used introduces serious temperature effects,
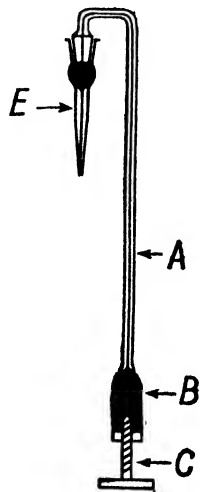necessitating elaborate precautions and making the burette hardly worth
while.

* Benedetti-Pichler, A. A., *Zeit. Anal. Chem.*, 1928, **73**, 200.
† Pauli, W., and Semler, A., *Kolloid Zeit.*, 1924, **34**, 145.
‡ Rehberg, P. B., *Biochem. J.*, 1925, **19**, 270.
§ Pincussen, L., *Biochen . Zeit.*, 1927, **186**, 28.
‖ Widmark, M. P., and Örskov, S. L., *Biochem. Zeit.*, 1928, **201**, 15.

For some estimations the presence of mercury in the burette may cause difficulties by reaction with the titrient. To avoid such effects some workers recommend the inclusion of a bubble of air between the solution and the mercury. The advantage so gained is more apparent than real, since the glass is wetted by the reagent. It follows, therefore, that after the first titration, the layer of liquid still adhering to the glass will have been in contact with a large area of mercury, and on refilling the burette some contamination is inevitable. To avoid this danger a burette of the syringe pattern can be used.

The micrometer syringe of Trevan has already been described in connection with micro-pipettes (p. 145). It is obvious that this instrument can be used as a burette without further modification, though some advantage is gained by substituting a capillary glass nozzle for the hypodermic needle
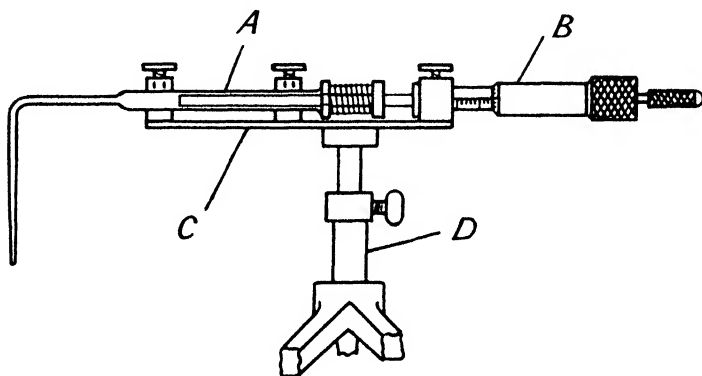
Fig. III.10.   Horizontal Micrometer Syringe Burette.

used in the original model. The capillary should be bent at right angles and the syringe used in the horizontal position. A similar arrangement is described by Hadfield.* This type of burette has the advantages of (a) high capacity in relation to the volume of liquid used in a single titration, (b) high overall accuracy, (c) an all-glass system.

Construction of this apparatus in the laboratory presents little difficulty, since all-glass tuberculin syringes and micrometer heads are readily obtainable. Fig. III.10 illustrates a burette of this type constructed by Wilson from a 1 ml. "VIM" tuberculin syringe A by sealing a length of capillary tubing to the needle spigot by means of bakelite cement. The piston, 5·05 mm. in diameter, is actuated by the "Shardlow" metric micrometer head B, so that a movement of one division (0·01 mm.) corresponds to 0·002 ml. The components are clamped to the metal bar C, which can be raised or lowered in the tripod stand D.

* Hadfield, I. H., *J. Soc. Chem. Ind.*, 1942, **61**, 45.

Holt and Callow* describe a simple horizontal tapless burette shown in fig. III.11, in which an air cushion is used to expel the contents.

Compression of the soft pressure tubing a by the screw clamps b forces the solution along the capillary tube c, which is suitably calibrated. This burette is rather difficult to control, the action being decidedly spongy, and in addition it is very sensitive to temperature variations during the titration.

Use of Micro-burettes. Micro-burettes should, as far as possible, be used with the tip immersed in order to avoid the uncertainty of drop-wise addition of the reagent. The drop formed is always greater than the diameter of the tip when the latter is wetted by the solution, thus setting a limit to the precision of the titration. Unless exceedingly fine jets are used it is difficult to make additions of less than 1 cu. mm.

If, however, the tip is immersed, it becomes quite practicable to make additions of 0·1 cu. mm. To avoid back diffusion into the tip, the latter should not be more than 0·3 mm. diameter.
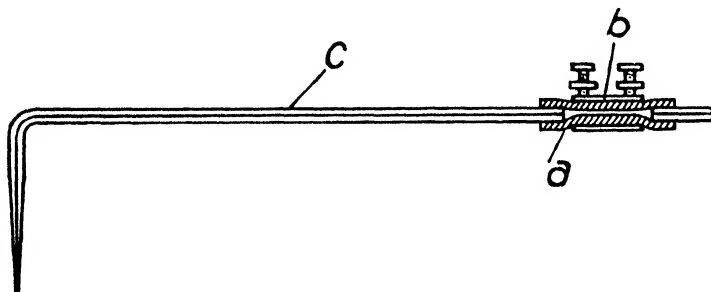
Fig. III.11. Holt and Callow's Horizontal Burette.

It is often an additional advantage to wax the tip, which can be done by one of two methods. With burettes open at both ends (e.g. Bang type, fig. III.5) the tip, which must be quite dry, is warmed and dipped into molten paraffin wax. The excess wax is then shaken off and a current of air blown through until the wax sets.

In closed burettes (i.e. those not fitted with detachable tips), a fine horse-hair may be inserted into the orifice before waxing. After shaking off excess wax and cooling the hair may be withdrawn, leaving the jet patent. Metal wire should not be used, since it is liable to scratch the inside of the glass, causing it to fracture on dipping into the hot wax.

Before commencing a titration it is, of course, essential to clean the burette thoroughly and to make sure that taps are properly greased and free from leaks. Badly fitting and badly greased taps are a prominent source of error with burettes of the Bang or Conway type.

* Holt, P. E., and Callow, H. J., *J. Soc. Chem. Ind.*, 1942, **61**, 99.

As far as is practicable the conditions of the titration should be so arranged that the end-point is not reached until the burette is at least one-third empty, except in the case of burettes such as the micrometer syringe. This practice ensures the "reading error" being insignificant compared with the volume of titrient used. It is advantageous always to start a titration from zero. This method causes no inconvenience with the Bang, Conway, or Rehberg burettes, but is not suitable in the case of the micrometer syringe. It has the merit of tending to cancel out errors in comparative titrations and often simplifies calibration of the burette.

When using vertical burettes, the precaution of limiting the rate of fall of the meniscus to 0·5 cm. per second should always be observed.

TABLE VII

SUMMARY OF MICRO-BURETTES

Type	Optimum Capacity	Delivery, etc.	Applications and Notes
Bang (p. 148).	1–10 ml.	Gravity, tap control. Vertical calibrated tube.	A good general-purpose burette, very suitable for routine titrations in aqueous media. Subject to drainage error in smallest sizes. Reagent easily protected from atmosphere. Grease may block jets.
Conway (p. 149).	0·1–1 ml.	Gravity, tap control. Horizontal calibrated tube.	Similar to above in lower volume range, but not subject to drainage errors.
Rehberg (p. 151).	0·2 ml. or less.	Positive delivery by screw-controlled mercury column. Vertical calibrated tube.	Easy to read, but filling tedious and causes wear on screw, leading to leakage of mercury. Can be made "ultra-micro" by using thermometer tubing. Solution not easily protected from atmosphere during filling. Mercury may be objectionable.
Trevan, Hadfield.	1 ml.	Positive delivery by piston and micrometer screw. Horizontal arrangement. All-glass system.	Easy to read and relatively large capacity with high precision if syringe of good quality. Compact, but micrometer liable to damage by corrosive agents. Very suitable for non-aqueous media. Somewhat tedious to fill, off-set by large capacity.

Calibration of Micro-volumetric Apparatus. All volumetric apparatus, whether purchased or "home-made," requires calibration before use if the highest precision is to be obtained. The method of calibration depends to some extent upon the type of apparatus, but in general the calibration should imitate the conditions under which the apparatus is to be used. It is, of course, essential that the apparatus should be perfectly clean before calibration is attempted.

Volumetric Flasks. In the larger sizes (5 ml. and above) the clean, dry flask should be weighed, filled with distilled water, adjusted to 20° C., stoppered, and reweighed. The weighing should be repeated at least twice more, drying the flask carefully without excessive heat in between. The volume is then easily calculated from the density of water at 20° C. Calibration of the smaller sizes of flask is more conveniently carried out with mercury. Owing to the convex meniscus, the volume of mercury contained in the flask will be less than the volume of water when the menisci are adjusted to the same mark. It is therefore necessary to add a correction to the observed volume as calculated from the weight and density of mercury. This correction varies with the diameter at the point of graduation, and can be determined from Table VIII.

TABLE VIII

Meniscus Corrections for Calibrating Tubes with Mercury

Tube Diameter at Graduation	Volume Correction	Tube Diameter at Graduation	Volume Correction
1·0 mm.	0·0005 ml.	3·5 mm.	0·0080 ml.
1·5 mm.	0·0015 ml.	4·0 mm.	0·0115 ml.
2·0 mm.	0·0025 ml.	4·5 mm.	0·0160 ml.
2·5 mm.	0·0040 ml.	5·0 mm.	0·0205 ml.
3·0 mm.	0·0055 ml.		

The figures represent the difference in the volume of mercury and water required to fill a vessel to a mark on a stem of the diameter indicated; thus a pipette "to contain" 0·2 ml. of water and 1·5 mm. bore would be calibrated with a weight of mercury equivalent to a volume of (0·2–0·0015 ml.)=0·1985 ml. This represents a difference of 19 mg. in the amount of mercury required.

Pipettes. Ordinary bulb pipettes are calibrated by weighing successive quantities of water as delivered from the pipette. A standard time of drainage should be adopted and the tip kept in contact with the vessel during this time.

If the pipette is to be used with a liquid such as alcohol, having appreciably different physical properties from water, then this substance should be used in the calibration. Pipettes designed "to contain" (e.g. "wash-out" pipettes) are best calibrated with mercury, correction for the convex meniscus again being applied. Pipettes delivering between two marks may be calibrated either with mercury or water. Again, mercury is the more satisfactory in the smaller sizes, but the pipette should be wet to allow for the film of moisture remaining on the walls in normal use. In this case no correction for the convex mercury meniscus should be applied, since

the error cancels out providing that the bore of the tube at the two graduations is the same.

Syringe pipettes of the Krogh pattern are readily calibrated by successive weighings, as with delivery pipettes. They are not subject to drainage errors or to errors arising from differences in viscosity or surface tension. Care should be taken, however, to see that no air bubbles are trapped in the syringe and, when emptying, no great pressure should be applied to the piston.

Dilution pipettes are calibrated "to contain" and mercury is again the best medium to use, but two calibrations are required, one for the total volume of the pipette and one for the volume of the stem. The dilution ratio is then given by:

$$\frac{\text{Volume of stem}}{(\text{Total volume} - \text{volume of stem})}.$$

Burettes. The neatest method of graduating burettes (or glass tubes generally) is by direct etching of the glass, but since this method requires a dividing engine or a screw-cutting lathe adapted for the purpose, it is not always convenient in the laboratory. However, equally good results can be obtained by the use of separate scales. The best type of scale is that etched on a glass mirror, since besides being almost unaffected by atmospheric conditions, each division with its reflection provides a ready means of avoiding parallax error. Paper scales are not very satisfactory because of their relative inaccuracy. They usually exhibit periodic errors, and unless thoroughly impregnated with some moisture-proof medium are liable to change their length with variations in humidity. In addition, the printed lines are usually unpleasantly thick.

It should be noted that Pyrex and similar glasses do not etch clearly, the lines being very diffuse. Consequently if the burette is of Pyrex glass the use of a separate mirror scale is to be preferred.

For the purpose of calibration, burettes may be divided into two classes: those depending on a graduated tube and those depending on a calibrated screw. With the graduated-tube types, the volume delivered between any two marks depends on the bore of the tube. It is seldom that a piece of ordinary tubing can be found which is truly uniform in bore along a sufficient length, though sometimes the variation is very slight. Recently, however, tested viscometer tubing has become available and should be of value.

Calibrated by Weighing. Vertical graduated-tube burettes such as Bang's are conveniently calibrated by weighing successive portions of mercury or water as delivered from the burette. If mercury is chosen, the burette must first be wetted with water and filled by forcing the mercury through the tip to avoid the trapping of air bubbles. If water is used, delivery should be made into a thin oil. Clock or typewriter oil is satisfactory, and the volume of each portion delivered should be about one-tenth of the total

volume of the burette. The actual volumes delivered, as calculated from the weights, are compared with the nominal values marked on the burette and a table of corrections is prepared, indicating the direction and magnitude of the error at each point. Water is more convenient than mercury for calibrating the Conway burette and must be used with burettes of the Rehberg pattern. An alternative method, used by Rehberg, is to measure the length of a weighed column of mercury at different parts of the tube. This method does not allow for the film of liquid retained on the wall of the burette and the mathematical treatment is somewhat complex.

The micrometer syringe may be calibrated by weighing water as described above.

Calibration by Titration. All types of burette may be calibrated by titration, a method which has much to recommend it, since it most closely resembles the conditions of use of the burette. Theoretically it is not so accurate as the weighing method, but providing a sufficiently sharp reaction is used, there is little difference in the final result.

The simplest and one of the most satisfactory reactions for the purpose is an acid-alkali titration using $2N$ solutions. The alkali should be in the burette and the acid prepared from weighed amounts of "constant boiling hydrochloric acid" (p. 161). The neutralised final solution should not be less than 0·1 molar.

The well-known iodate-iodine reaction (compare p. 90) can also be used.

EXPERIMENTAL TECHNIQUE IN MICRO-TITRATION

Since, as explained in preceding pages (pp. 130–141), the majority of titration errors are functions of concentration, the micro-analyst should not work with highly diluted solutions, but should preferably use reagents of the same order of concentration as does the macro-analyst. It is more accurate to titrate with $N/100$ solutions from a 2 ml. micro-burette than to use $N/1,000$ solutions from a 25 ml. burette.

Attention should be paid to drop-size during titration and apparatus should be chosen to suit the experimental conditions in accordance with the recommendations summarised on Tables VI and VII.

As explained on pp. 153–154, it is best when titrating to have the tip of the burette almost touching the surface of the liquid; very small drops of liquid may be released by bringing the surface of the solution in the titration vessel into contact with the tip of the burette. The capillary tips of small micro-burettes should be placed within the titrated liquid.

Titrations should only be carried out in a good light and when the operator's eyes are well accommodated to the light. A white background and foreground are both essential, and the lighting must be arranged so that shadows are avoided and the operator is not dazzled (see p. 6). For comparison purposes the titration vessel should be held at about 15–18 in. below eye-level. Micro-titrations with adsorption indicators require special care, particularly when fluorescent indicators are used. Since the end-point in these cases is due to a change of colour in a turbid mixture it is often advisable to titrate in a darkened room and view the flask against a darkened background in a beam of transmitted light.

The end-point of a titration in a micro-chemical work must be very carefully judged. Where the operator is unfamiliar with the colour change, the "comparison tube method" is recommended. This consists of setting up alongside the titration vessel a similar tube containing an equal volume of distilled water. The same amount of indicator is added to both the titration vessel and the comparison tube and a measured drop of the standardised reagent is added to the comparison tube so as to produce the necessary indicator colour change. The unknown solution is then titrated until the colour is identical in tinge with that of the comparison tube, when both are viewed from above through the thickness of the liquid. In the subsequent calculation the volume of reagent added to the blank tube is subtracted from the observed titration figure. This method is very satisfactory and should be used whenever the colour change is not abrupt. For more objective results solutions may be titrated to a null point on a

photo-electric instrument (see Part IV, pp. 244–249), care being taken, of course, to see that the volumes of liquid in the two tubes are identical.

It is a good technique for the analyst to familiarise himself with volumetric end-points by blank titrations and by titrating known volumes of a comparison reactant solution. Some idea of the limits and reproducibility of the analytical method is then obtained. In this way, too, one can judge the minimum amount of indicator which may safely be added. Bearing in mind that since the indicator is itself a reagent which enters into the reaction (see p. 131), the amount added should be as small as possible both in volume and concentration, compatible with a sharply visible colour change. In view of the chemical errors that may be associated with absorption or escape of volatile products (e.g. carbon dioxide) titrations should be carried out as rapidly as possible.

Erlenmeyer flasks of 10–50 ml. capacity are most useful for micro-titrations, but often a centrifuge tube or a still smaller vessel must be used. Mixing of solutions by swirling between additions of reagent is quite easy in an Erlenmeyer flask; with narrow tubes this is not possible, but a small glass rod drawn to a point with a small knob on the upper end is a convenient form of stirrer.

A much better procedure is to pass air through the solution by means of a fine capillary jet, when the regular stream of bubbles causes adequate mixing. This cannot be done when oxidation titrations are being performed, in which case an inert gas such as nitrogen should be used. The error due to inadequate mixing of the solution cannot be too strongly stressed in

Fig. III.12.
Micro-titration in the Absence of Air.

micro-analysis. Particular care should be taken to see that a drop of the liquid to be titrated or added is not left as a bead on the side of the vessel.

Sometimes titrations must be made in the absence of air. A container with a cork bored with three holes is then used (compare fig. III.12). Into one fits the tip of the burette, whilst the others serve as an inlet and outlet for an inert gas such as nitrogen (washed through pyrogallol or similar reagent) or carbon dioxide. (Carbon dioxide cannot be used if pH considerations apply.) The inlet tube is capillated and projects almost to the bottom of the container. In order to sweep out the air from above the liquid, as well as from solution, the gas stream should be allowed to flow for some time (about 10 minutes) before titrating, and should be maintained during the titration to ensure adequate mixing of the solution.

The precautions to be taken in reading a micro-burette have been given on pp. 153 and 156, but as a final rule it should be made a strict routine *always* to apply any calibration correction to the titration figure and to record the corrected value *immediately* in a permanent note-book and not on an odd scrap of paper.

ILLUSTRATIVE EXAMPLES OF MICRO-VOLUMETRIC PROCEDURES

1. MICRO-ANALYSIS INVOLVING ACID-ALKALI TITRATION

Acid-alkali titrations are particularly adaptable to micro-analytical methods. Whilst the apparatus to be used depends, of course, on the particular analytical process, the previous section (pp. 146–154) should prove helpful in the selection of the type of burette to be used in the final titration. The experimental method of approaching and defining the end-point should be in accordance with the suggestions given on pp. 158–160.

The theoretical considerations discussed on pp. 131–137 should be borne in mind when carrying out this type of analysis. Since the attainment of a sharp end-point necessitates an abrupt pH change, the standard reagent used in the micro-burette should be a strong acid or a strong base, such as hydrochloric acid or caustic soda, rather than a weak acid or a weak base, such as an organic acid or an alkali carbonate. As a primary standard, hydrochloric acid prepared by dilution from a constant-boiling solution is to be recommended.*

The choice of indicator will depend upon the ionisation constant of the acid and alkali concerned, and reference should be made to the Tables on pp. 132–135 for indicators operating within specified pH limits. Various types of acid-alkali titrations are exemplified more fully by the examples of important micro-analyses which are given below.

A. MICRO-TITRATION OF A STRONG ACID AND A STRONG BASE

This is an acid-alkali titration of the simplest type, since the reacting solution is almost unbuffered and the pH changes over several units in the region of the end-point. The selection of the indicator should therefore be made primarily on grounds of colour sensitivity: screened indicators (p. 134) are particularly useful.

Example: The Estimation of Urea in Blood.†

PRINCIPLES. Urea is converted to ammonium carbonate by the action of urease. Excess sodium carbonate is added and the liberated ammonia is aspirated into an excess of standard acid. The excess of acid is back-titrated with standard alkali.

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* Vogel, I. A., "Text-book of Quantitative Inorganic Analysis," p. 279 (Longmans, London, 1943).
† Van Slyke and Cullen, *J. Biol. Chem.*, 1916, **24**, 117.

SOLUTIONS.

1. *Urease* (Dunning). B.D.H., London.

2. *Phosphate buffer.* Dissolve 6 g. of KH_2PO_4 and 2 g. of anhyd. Na_2HPO_4 in 1 l. of water.

3. $N/50$ *hydrochloric acid.*

4. $N/50$ *sodium hydroxide.* Keep in a paraffined bottle and prepare each week from $N/10$ solution.

5. *Saturated potassium carbonate.* Dissolve 900 g. of K_2CO_3 in 100 ml. of water.

6. *Caprylic alcohol.*

7. *Screened methyl red* (p. 86).

METHOD. 1·00 ml.* of serum or plasma is placed in tube A (fig. III.13) and about 5 mg. of crushed urease tablet and 1 ml. of phosphate buffer are added. The tube is stoppered and placed in an incubator for 15 minutes
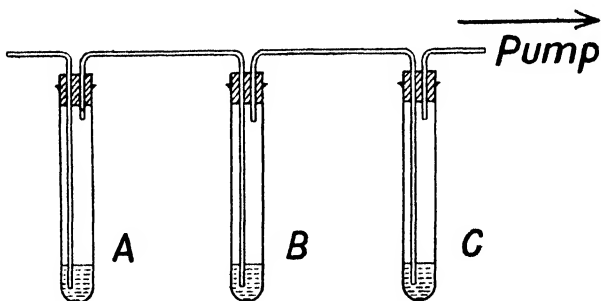
Fig. III.13.   Aspiration Apparatus for Estimation of Urea.

(temperature tolerance 25° and 50° C.) in order to convert the urea into ammonium carbonate. Prolonged contact with red cells tends to liberate ammonia from arginine by the action of arginase in the urease. 1 ml. of saturated potassium carbonate is added to tube A, and immediately A is connected up to tube B, which contains 2·00 ml. of $N/50$ hydrochloric acid and 3 drops of indicator, and thence by way of tube C which contains 1·00 ml. of acid, 1 ml. of water, and 3 drops of indicator, to a suction pump.

The ammonia is gently aspirated from A to B, very slow bubbling being used initially, since the bulk of the ammonia comes over in the first minute, although it is not completely removed in less than 15 minutes' aspiration. Should the indicator change from purple to green during the aspiration, a further 2·00 ml. of standard acid should quickly be added to tube B.

The excess acid in tubes B and C is then titrated with $N/50$ caustic soda from a micro-burette, the end-point being reached when a sharp colour

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* When, as above, volumes are given to two or more significant figures accurately standardised micro-pipettes should be used.

change from purple to green occurs. Each millilitre of $N/50$ acid is equivalent to 0·6 mg. of urea, and in normal blood the titration is of the order of 0·5 ml.

B. Micro-titration of the Salt of a Weak Base with a Strong Base

Titrations of this class, in which the strong base is placed in the burette, have end-points in the pH range 8–12, and an indicator such as phenolphthalein should be used. Under these circumstances the error due to absorption of atmospheric carbon dioxide (see p. 136) may be serious and should be specially guarded against, e.g. by bubbling a stream of carbon dioxide-free air through the titrated solution.

Example: Estimation of Sulphate as Benzidine Sulphate.

Principles. Benzidine sulphate is very insoluble in water and in acids. In hot solution benzidine sulphate is readily decomposed by caustic soda, excess of which is indicated by phenolphthalein.

The application of this method to sulphate estimation in blood serum* illustrates the procedure to be followed when very small quantities of sulphate are to be estimated. Centrifuge technique, the use of acetone to depress the solubility of the salt, and the employment of a continuous air stream for mixing during titration are all experimental features to be noted.

Solutions.
1. *Benzidine base.* 1% solution in acetone.   ·
2. *Trichloracetic acid,* 20%.
3. *Pure acetone.*
4. *$N/50$ caustic soda.*

Method. 2·0 ml. of serum are added, in a centrifuge tube, to 2·0 ml. of water; 2·0 ml. of 20% trichloracetic acid are added; after mixing a further 4·0 ml. of water are added and the contents are again mixed. The tube is then centrifuged and 5·0 ml. of the clear supernatant liquor are transferred to another 15 ml. centrifuge tube. To this are added 10 ml. of benzidine solution, and the tube is then capped, placed in ice-water for 60 minutes to allow of flocculation, and then centrifuged for 30 minutes (keep cap in position to avoid loss of acetone by evaporation). The supernatant liquid is then poured off and the tube is inverted over filter-paper to drain (see p. 34). The precipitate is then broken up by tapping the tube, 15 ml. of acetone are added, and the tube is again centrifuged for 15 minutes at 3,000 r.p.m. After removal of the wash-liquid, the tube is again drained over filter-paper for 5 minutes.

* Cope, C. L., *Biochem. J.*, 1931, **25**, 1183.

To the precipitate are now added 0·5 ml. of distilled water and 1 drop of phenolphthalein on the end of the glass stirring rod (fig. III.14). The centrifuge tube is then fitted into the steam jacket and the whole is arranged so that the tip of a Rehberg burette dips just below the surface of the liquid. A gentle stream of carbon dioxide-free air is then passed into the liquid and the titration is commenced. The steam is turned on when the pink colour of the indicator tends to be persistent, and the titration is finished as rapidly as possible in order to avoid heating of the micro-burette. At the end of the titration the air stream is increased so as to splash up liquid and wash the sides of the tube. More alkali is then added if necessary.

The burette is then removed from the tube and allowed to return to room temperature before the meniscus reading is finally taken. A blank titration is necessary to determine the amount of alkali required to give an equivalent pink end-point with 0·5 ml. of distilled water at 100° C., and subtraction is made accordingly.
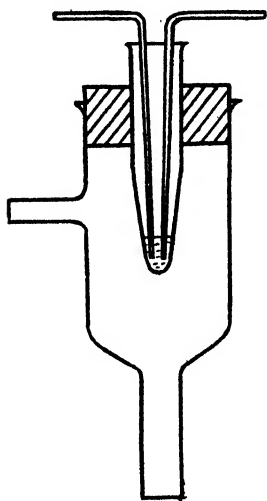
Fig. III.14.
Micro-titration of Benzidine Sulphate Inside Steam Jacket.

C. MICRO-TITRATION WITH A STRONG ACID IN THE PRESENCE OF A VERY WEAK ACID

In titrations of this class the titrated solution becomes highly buffered and the selected end-point should not depend significantly on the concentration of the weak acid which neutralises part of the strong base. As explained on pp. 131-136, pK_{HIn} should therefore be 2–3 units above the value of pK_a of the weak acid (i.e. on the more alkaline side). Thus when, as in the example, phosphoric acid (pK_a for $Na_2HPO_4=7·2$) is the weak acid, thymolphthalein ($pK_{HIn}=9·2$) is a suitable indicator. The comparator method of titration (p. 158) is helpful and leads to enhanced accuracy.

Example: The Estimation of Phosphate in Organic Matter.[*]

PRINCIPLES. Phosphate is precipitated as ammonium nitro phosphomolybdate, $(NH_4)_3PO_4,12H_2MoO_4,2HNO_3$, dissolved in excess alkali, heated to remove ammonia, and the excess alkali is titrated with standard acid. The complex contains 26 replaceable hydrogen atoms, besides 3 ammonia groups; thus 1 equivalent of phosphate titrates with 28 of alkali, according to the equation:

$$H_3PO_4,12H_2MoO_4,2HNO_3+28NaOH = Na_2HPO_4+12Na_2MoO_4+2NaNO_3.$$
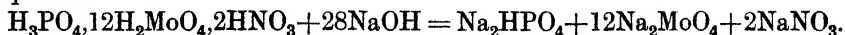
This is an example of micro manipulation using N solutions for titration.

[*] Neumann, *Zeit. Physiol. Chem.*, 1902, **37**, 115.

SOLUTIONS.

1. *Ammonium nitrate*, 60%.

2. *Molybdate solution.* A 10% aqueous solution of pure ammonium molybdate.

3. *N sodium hydroxide.*

4. *N sulphuric acid.*

5. *Thymol-phthalein indicator.*

METHOD. Organic material containing about 1 mg. of P is oxidised with sulphuric and nitric acids in a Kjeldahl flask (p. 100) or by the ammonium nitrate method (p. 101). The residue is dissolved in a little water and neutralised with ammonia-water until it is just pink to methyl red indicator (more alkaline than this may cause precipitation of calcium phosphate).

The solution is transferred quantitatively to a small (50 ml.) beaker, 2·5 ml. of 60% ammonium nitrate are added, and then water to a total of 10 ml. The beaker is then placed on a water-bath and warmed to about 60° C. 4 ml. of warm ammonium molybdate solution are then added, drop by drop with stirring, and the mixture is maintained at 60°–65° for about 15 minutes. It is then filtered by the filter-stick procedure (see p. 21). The beaker and precipitate are washed three times with 2·5 ml. of 1% ice-cold potassium nitrate, care being taken to wash down the sides of the beaker with the solution and to drain completely between washings.

To the beaker are now added 5 ml. of water and exactly 2·00 ml. of *N* sodium hydroxide (carbonate-free). The beaker is brought to the boil and the filter-stick is cleared of precipitate by fixing a rubber bulb to the end of the stick and drawing hot liquid through the precipitate and then forcing it out again a number of times. The liquid is then boiled again to remove ammonia completely.

A few drops of thymol-phthalein indicator are added: the solution should appear blue, showing excess of alkali; more alkali should be added and the boiling repeated if this is not the case. After cooling out of contact from air, the solution is back-titrated with N H_2SO_4 from a micro-burette until the blue colour is just discharged. The volume of acid required is subtracted from the alkali used and the resultant multiplied by 1·11 to give milligrams of P in solution. This factor may, however, be modified according to particular conditions,* and should therefore be verified by using a standard solution of phosphate.

D. MICRO-TITRATION INVOLVING THE REPLACEMENT OF A WEAK ACID BY A STRONG ACID

Titrations of Group D differ from those of Group C in that the weak acid is liberated completely from its salts, and consequently pK_{HIn} should be

* Errors due to co-precipitation may arise.

2–3 units *less* than pK_a. Indicators which change colour at low pH must be used, and since under these circumstances the overall change of pH in the vicinity of the stoichiometric end-point is small, careful colour matching by the comparison tube method should always be used.

Example: The Estimation of Total Organic Acids in Urine.*

PRINCIPLES. Phosphates, carbonates, etc., are removed by lime treatment. The filtrate, which contains the organic acids as calcium salts, is neutralised with acid to phenolphthalein end-point and titrated from pH 8·0 to pH 2·5 with standard HCl, whereby the buffering effect of each molecule of the weak acid is almost equivalent to 1 molecule of HCl.

SOLUTIONS.

1. *Powdered calcium hydroxide shaken up to a 20% suspension.*
2. *Hydrochloric acid, 10%.*
3. *$N/10$ hydrochloric acid.*
4. *Phenolphthalein indicator.*
5. *Bromo-thymol blue indicator, 0·04%.*

METHOD. 8·0 ml. of urine are pipetted into a test-tube and 2·0 ml. of lime solution are added. The mixture is thoroughly shaken for 5 minutes, allowed to stand for 15 minutes, and then filtered twice through the same filter-paper. 5·0 ml. of the filtrate are transferred to a 10 ml. Nessler tube, 2 drops of phenolphthalein added, and the contents are neutralised with 10% HCl until the pink colour just disappears. Into a similar Nessler tube are placed 5 ml. of water, 2 drops of phenolphthalein, and 2 drops of dilute caustic soda solution. This is also neutralised with 10% HCl until the pink colour just disappears. To this comparison tube is added exactly 1·0 ml. of $N/10$ HCl; to both tubes is then added 1 ml. of bromothymol blue indicator.

The two tubes are placed together on a white tile and the unknown solution is titrated with $N/10$ HCl until it exhibits exactly the same degree of red colour as the blank tube when the two tubes are placed side by side and viewed from above.

From the titration figure must be subtracted 1·0 ml., which was added to the control; the resultant represents millilitres of $N/10$ acids in 4 ml. of urine.

In this process creatinine is also titrated and allowance must be made accordingly (11·32 mg. of creatinine titrates with 1 ml. of $N/10$ HCl).

2. OXIDATION-REDUCTION TITRATIONS: INORGANIC REAGENTS

Most of the standard inorganic oxidising agents can be used microchemically. The experimental technique is amply illustrated by one or

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* After Van Slyke and Palmer, *J. Biol. Chem.*, 1920, **41**, 567.

two examples of the use of each reagent, for analogous oxidation-reduction titrations can be modified similarly to suit the requirements of micro-analysis.

The Rehberg type of micro-burette (p. 151), in which mercury comes in contact with the standard solution, should not be used with strong oxidisers, such as potassium permanganate or ceric sulphate; horizontal burettes of the Conway or Wilson types (pp. 149-150) can be used conveniently.

A. MICRO-TITRATION WITH POTASSIUM PERMANGANATE

Highly dilute solutions of $KMnO_4$ are none too stable, and should be prepared by diluting a $N/10$ solution immediately before use. It is, of course, essential to work in the presence of excess of mineral acid, preferably H_2SO_4, so that reduction to the manganous salt is complete. The presence of chlorides should be avoided as much as possible.

Example 1, the micro-chemical estimation of calcium, has been chosen to illustrate the removal of proteins and also the use of centrifuge technique in purifying a precipitate before analysis. The use of manganous sulphate as a potential mediator may be noted.

Example 2, the estimation of potassium as cobaltinitrite, is a further example of a precipitation analysis in which it is essential to adhere to a rigid analytical technique and to control operations by carrying out a blank analysis.

Example 1: The Estimation of Calcium (e.g. in Blood*).

PRINCIPLES. Blood proteins are precipitated with trichloracetic acid. The protein-free filtrate is buffered with sodium acetate prior to the precipitation of calcium as the oxalate. The separated calcium salt is dissolved in acid solution and titrated with standard permanganate.

The general application of this method for estimating calcium needs care.† In the presence of a large excess of magnesium co-precipitation is apt to occur. Under such circumstances it is advisable to precipitate both Ca and Mg with ammonium phosphate, dissolve the precipitate in sulphuric acid and separate the calcium as sulphate by the addition of alcohol. This precipitate is then redissolved and estimated by the oxalate method.

SOLUTIONS.

1. *Trichloracetic acid*, 20%.

2. *Sodium acetate*, 20%.

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* Kramer, B., and Tisdall, F., *J. Biol. Chem.*, 1921, **48**, 223; Stanford, R., and Wheatley, A., *Biochem. J.*, 1925, **19**, 710.

† Cf. Kolthoff and Sandell, "Text-book of Quantitative Inorganic Analysis" (Macmillan, New York, 1936); Sandell, "Colorimetric Determination of Traces of Metals" (Interscience Publishers, New York, 1944).

3. *Bromocresol green*, 0·02%.

4. *Ammonia-water*, 1 : 1.

5. *Saturated ammonium oxalate.*

6. *N sulphuric acid.*

7. *N/100 potassium permanganate.*

8. *Manganous sulphate*, 5%.

9. *Calcium oxalate, saturated.* 15 ml. when titrated as below should not take more than 0·2 ml. of $N/100$ permanganate.

METHOD. 4·0 ml. of plasma are mixed with 12·0 ml. of water and then 4·0 ml. of trichloracetic acid are added. After standing for 5 minutes the mixture is filtered through ash-free filter-paper into a clean tube. 10·0 ml. of clear filtrate are pipetted into a 15 ml. centrifuge tube, 1 ml. of acetate solution and a few drops of bromocresol green indicator are then added, followed by 1 ml. of saturated ammonium oxalate. The mixture is stirred with a glass rod and 50% ammonia is added drop by drop until the indicator is of colour equivalent to pH 5·0. The tube is allowed to stand for 1 hour to complete precipitation and then is centrifuged. The supernatant liquid is then removed by means of suction through a capillary with its end bent through an angle of 180° (see fig. I.19, *d*), care being taken to ensure that the tip of the capillary is always just below the meniscus of the liquid, until only 0·2 ml. of liquid is left in the tube. The precipitate is broken up by tapping and then 10 ml. of saturated calcium oxalate solution are added down the sides of the tube. The tube is again centrifuged and the supernatant solution is removed as before: this washing process is repeated a second time and the supernatant liquid is again removed.

The precipitate is then broken up and dissolved in 2 ml. of N sulphuric acid. To the solution is then added 1 ml. of manganous sulphate solution, and the tube is placed on a water-bath warmed to about 40° C. $N/100$ permanganate is then run in from a micro-burette until there appears a faint pink colour which persists for 1 minute. With each batch a blank titration, taken to the same intensity of pink colour, is carried out on all the reagents as used. This blank is subtracted from the actual titration figure.

Example 2: The Micro-chemical Estimation of Potassium.*

PRINCIPLES. After ashing or wet oxidation of organic matter and removal of heavy metals in the usual way, potassium is precipitated on addition of sodium cobaltinitrite as the sodium-potassium double salt. The washed precipitate is dissolved in dilute acid in the presence of excess standard permanganate until nitrite is oxidised. The excess of permanganate is

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* Tisdall, F., and Kramer, B., *J. Biol. Chem.*, 1921, **46**, 339; Van Rysselberge, P., *Ind. Eng. Chem.* (Anal. Edn.), 1931, **3**, 3.

then removed by addition of standard oxalate, which is in turn titrated with standard permanganate to an end-point.

SOLUTIONS.

1. *Sodium cobaltinitrite.* (*a*) Dissolve 25 g. of cobalt nitrite in 50 ml. of distilled water.   Add 12·5 ml. of glacial acetic acid.   (*b*) Dissolve 120 g. of sodium nitrite in 180 ml. of distilled water.

Add the whole of (*a*) to 210 ml. of (*b*) and aspirate air through the mixed solution until nitrous fumes cease to be evolved.   Allow to settle overnight and filter before use.

2. *Sodium nitrite, 20% solution.*

3. *Glacial acetic acid.*

4. $N/100$ *potassium permanganate.* Dilute from $N/10$ solution as required.

5. $N/100$ *sodium oxalate.* Also dilute as required.

METHOD. The sample, which should contain potassium of the order of 0·05--0·5 mg., is ashed in a silica crucible at just red heat or treated by the wet oxidation procedure described on p. 100.   In either manner the ammonium salts which would precipitate with potassium are volatilised away.   The sample is eventually contained in about 4 ml. of neutral solution in a centrifuge tube.

If the sample contains very little organic matter (e.g. urine) then ammonium salts may be removed as follows.   To the solution in a centrifuge tube is added 0·5 ml. of 20% sodium nitrite solution followed by 0·2 ml. of glacial acetic acid.   The tube is then placed in a boiling water-bath for about 5 minutes.   Ammonium salts and urea are decomposed thereby and nitrogen gas is liberated.

To the cooled solution in the centrifuge tube are then added, drop by drop with shaking between additions, 2 ml. of the cobaltinitrite reagent, and the tube is allowed to stand for about 30 minutes.   The manner in which this precipitation is carried out is of vital importance, and a rigid technique must always be followed, since the composition of the salt may be varied by changing the manner of addition of the reagent.   Theoretically the precipitate is $K_2NaCo(NO_2)_6$, but in practice some $K_3Co(NO_2)_6$ also precipitates, the amount of the latter depending upon the circumstances of the precipitation.   Temperature and speed of addition of reagent are the dominating factors, and each analyst is therefore advised to carry out titrations on standard potassium solutions which have been subjected to a rigorous precipitation technique, and to establish for himself the titration factor.

After precipitation is complete, the tube is centrifuged at high speed for 5 minutes and all but 0·2 ml. of the supernatant fluid is removed by suction *via* an upturned capillary tube pipette.   5 ml. of water are then added to

the tube, which, after mixing, is again centrifuged, and the supernatant fluid is again removed. This washing process is repeated, and finally the wash liquid is removed completely.

The precipitate is now broken up, by gently tapping the bottom of the tube, and an excess of $N/100$ permanganate is added from a micro-burette ($2 \cdot 0$ ml. is sufficient if the potassium is of the order of $0 \cdot 2$ mg.) followed by about 2 ml. of $4N$ H_2SO_4. The contents of the tube are then mixed, using a fine glass rod, and the tube is placed in a boiling water-bath for 1 minute, when the solution should be clear and still pink. If it is cloudy, then heating should be continued, with addition of more permanganate if necessary, until the yellowish turbidity is gone and a clear pink solution persists.

Overheating at this stage should be avoided, since it leads to precipitation of manganese dioxide and so increases the titration.

An excess of $N/100$ oxalate is then measured accurately into the solution to destroy the pink colour, and the titration is completed by adding $N/100$ permanganate from a micro-burette until, on looking through the depth of the liquid, a faint colour persists.

CALCULATION. From the total amount of permanganate is subtracted the equivalent of the oxalate used : the remainder is multiplied by the analyst's predetermined factor. Theoretically 1 ml. of $N/100$ $KMnO_4 = 0 \cdot 065$ mg. K, but usually the figure is nearer to $0 \cdot 070$ mg. K.

B. MICRO-TITRATION WITH CERIC SULPHATE

Ceric sulphate has found its place as a valuable oxidising agent, mainly due to the work of Furman and his collaborators.* It must be used in $0 \cdot 5$–$2N$ acid solution, and, in contrast to permanganate, can be employed safely in the presence of hydrochloric acid. The iron complexes of o-phenanthroline or of $\alpha\alpha'$-dipyridyl are convenient internal indicators :

$$2Ce(SO_4)_2 + 2FeSO_4 = Ce_2(SO_4)_3 + Fe_2(SO_4)_3$$
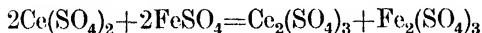
Standard ($N/10$) solutions of ceric sulphate may be prepared by dissolving weighed quantities of either pure anhydrous ceric sulphate, $Ce(SO_4)_2$, or ceric ammonium sulphate, $Ce(SO_4)_2,2(NH_4)_2SO_4,2H_2O$ in N sulphuric acid. Arsenious oxide, iron, potassium iodide, or potassium ferrocyanide may be used for standardisation.

Diluted ceric sulphate solutions are not stable for more than a few hours.

Since an internal oxidation-reduction indicator is required, it is necessary, on the micro-chemical scale, to carry out a blank titration with the reagents used.

* See Furman's review in "Newer Methods of Volumetric Chemical Analysis," edited by W. Bottger (Chapman and Hall, London, 1938).

Example: Micro-determination of Iron.*

PRINCIPLES. Iron, after separation, is dissolved in HCl and passed through a silver reductor. $\alpha\alpha'$-dipyridyl indicator is added and the solution is titrated with ceric sulphate until the red colour of the ferrous dipyridyl complex has gone completely.

The iron precipitate is best dissolved in HCl, since H_2SO_4 tends to inhibit the formation of the dipyridyl complex.

The titration should be carried out as quickly as possible, since re-oxidation tends to occur in the ferrous solution.

When a silver reductor is used the deleterious formation of peroxides is reduced to a minimum. For this reason the more common Jones' reductor (amalgamated zinc) is not suitable for micro-analysis.

REAGENTS.

1. *N hydrochloric acid.*

2. *$\alpha\alpha'$-Dipyridyl.* 0·25 g. in 100 ml. of 15% ammonia water.

3. *Standard ceric sulphate.* $N/100$ in N sulphuric acid.

4. *Silver reductor.* Dissolve 10 g. of silver nitrate in $N/50$ nitric acid. Add a strip of copper foil and allow reaction to proceed until all the silver is precipitated. Wash the silver metal with dilute H_2SO_4 until the copper is removed and then transfer to a tube 20 cm. long by 7 mm. diameter, fitted at the top with a funnel and at the bottom with a tap and capillary tube attachment.

METHOD. The iron to be estimated (0·1–1·0 mg.) is separated from other metals in the usual manner (see C, p. 172, below) and finally dissolved in 20 ml. of N HCl. The solution is then poured through the silver reductor at a moderately slow rate and allowed to fall into a 100 ml. narrow-mouthed Erlenmeyer flask through which is passing CO_2, to exclude oxygen. The reductor tube is rinsed through with 10 ml. of N HCl, which is allowed to fall into the Erlenmeyer flask. With the CO_2 still passing, to the solution are added 5 drops (0·1 ml.) of $\alpha\alpha'$-dipyridyl solution, and ceric sulphate is then added from a micro-burette until the red colour is just discharged. A blank is run by passing 30 ml. of N HCl through a similar silver reductor, adding the indicator, and titrating with $N/100$ ceric sulphate. This blank should be subtracted from the previous reading.

C. MICRO-TITRATION WITH POTASSIUM DICHROMATE

The example is chosen to illustrate the precision in technique that is needed when applying a well-known volumetric process to micro-analysis.

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* Walden, G., Hammett, L., and Edmonds, S., *J.A.C.S.*, 1934, **56**, 350; Van Nieuwenberg, C., and Blumendahl, H., *Microchem.*, 1935, **18**, 39; Colson, A. F., *Analyst*, 1945, **70**, 255.

Example: Micro-determination of Iron.*

PRINCIPLES. Iron is taken into solution with hydrochloric acid, reduced with stannous chloride, and titrated with dichromate using diphenylamine indicator.

SOLUTIONS.

1. *Ferric chloride, 5%, in 6% sulphuric acid.*

2. *Syrupy phosphoric acid, 7%.*

3. *Stannous chloride, 1%, in concentrated hydrochloric acid.*

4. *Mercuric chloride saturated solution.*

5. *Diphenylamine, 0·1% solution in concentrated sulphuric acid.*

METHOD. The sample containing iron (0·2-0·5 mg.) is dissolved in hydrochloric acid. Group II metals are removed as sulphides in acid solution and the filtrate is evaporated down to remove H_2S. It is then oxidised with nitric acid, excess of which must be removed by evaporation with sulphuric acid. The solution is transferred to a 15 ml. centrifuge tube, treated with ammonium chloride and ammonia, and the precipitate is centrifuged and washed. Any chromium is converted to chromate by peroxide treatment and then washed out.

The ferric hydroxide precipitate is dissolved in 1 ml. of dilute hydrochloric acid; the solution is brought to boiling and stannous chloride is added, drop by drop, until the yellow colour fades, showing complete reduction of iron to the ferrous state. One drop of stannous chloride is then added in excess and the sides of the tube are washed down with 0·5 ml. of boiled distilled water.

The tube is then connected to a carbon dioxide generator and the pure gas is passed through the solution for 5 minutes. 0·2 ml. of mercuric chloride is then added to destroy the excess of stannous chloride. Mercurous chloride precipitates as a silky white precipitate. (The appearance of a black precipitate of mercury indicates that the reduction has proceeded too far, due to addition of great excess of stannous chloride, and this invalidates the estimation.)

The passage of carbon dioxide is continued; 1·5 ml. of ferric chloride solution, followed by 1 ml. of phosphoric acid and 2 drops of the diphenylamine indicator are added, and the solution is titrated with $N/250$ dichromate from a micro-burette until a persistent blue-violet colour forms. It is most necessary to conduct a blank experiment using all the reagents mentioned above (but without the ferrous salt) to determine the amount of dichromate necessary to produce the same degree of colour change. This value must be deducted from the titration figure of the iron solution before proceeding to the calculation.

* Knop, J., *J. Amer. Chem. Soc*, 1924, **46**, 263.

D. MICRO-TITRATION WITH STANDARD IODINE

The wide micro-chemical applicability of standard iodine is obvious. The following precautions should be taken: (i) the titration should be carried out at room temperature; (ii) the starch solution should be fresh and checked for sensitivity; (iii) pH control is more essential than in corresponding macro-analysis.

Example 1: The Micro-estimation of Arsenic after Separation as Arsine.*

PRINCIPLES. Organic matter is oxidised with nitric/sulphuric acids. If much arsenic is present it is distilled off as arsenic trichloride. The distillate (or the acid digest in cases where traces are present) is then treated with zinc, and the arsine gas evolved is passed into silver nitrate solution. The arsenite which forms is titrated with standard iodine.

The method is very satisfactory for from 5 µg.–50 µg. of arsenic; antimony does not interfere under these conditions.

SOLUTIONS.
1. *Nitric acid and sulphuric acid, both arsenic-free.*
2. *Saturated ammonium oxalate.*
3. *Stannous chloride, 40%, in hydrochloric acid.*
4. *Copper sulphate, 5%.*
5. *N/50 silver nitrate.*
6. *10N sodium hydroxide.*
7. *N/200 iodine solution.*

METHOD. A sample (which should contain between 5 µg. and 50 µg. of arsenic) is transferred to a Kjeldahl flask, treated with about 10 ml. of nitric acid, and heated gently at first until frothing ceases and then more vigorously until solution is complete. Heating is continued, with addition of nitric acid as necessary, until the bulk of the chlorides have been removed, mainly as nitrosyl chloride. This procedure of preliminary digestion with nitric acid alone is essential to avoid possible loss of arsenic as the trichloride.

About 10 ml. of sulphuric acid are then added and heating is continued until oxidation of organic matter is complete, more nitric acid being added from time to time to assist in oxidation. The contents of the flask are then evaporated until sulphuric fumes appear, cooled, treated with 20 ml. of water, and again taken down to the fuming stage. The cooled residue is dissolved in 20 ml. of water and 1 ml. of saturated ammonium oxalate solution is added. Heating is again continued to fuming, and in this way the residual nitrogen compounds are destroyed. If the sample contains more than 50 µg. of arsenic it is distilled according to the Bang procedure (see below, p. 174); if less, then it is dealt with as follows:

* Levvy, G., *Biochem. J.*, 1943, **37**, 598.

The oxidised solution is diluted with water to about 40 ml. so that the concentration of sulphuric acid is reduced to about 20%. It is transferred to a 100 ml. Erlenmeyer flask and attached to an absorption train composed of a scrubbing tube containing $10N$ caustic soda to remove sulphides and a special tube (see fig. III.15) containing 1·5 ml. of $N/50$ silver nitrate solution.

To the solution in the Erlenmeyer are added 3 drops of 5% copper sulphate, 10 drops of 40% stannous chloride, and 15 g. of zinc shot. The stopper of the Erlenmeyer is forced home and the evolved gases are allowed to bubble through the absorption-tubes until effervescence ceases. The silver solution is then titrated with $N/200$ iodine from a Conway burette (see p. 149). Since silver ions would interfere with the titration, a crystal of potassium iodide is added prior to the addition of the iodine. The silver iodide precipitate which first appears redissolves in the excess of potassium iodide.

About 3 g. of sodium bicarbonate, followed by a drop of starch solution, are then added, and standard iodine is run in from the burette until a persistent blue tinge appears.
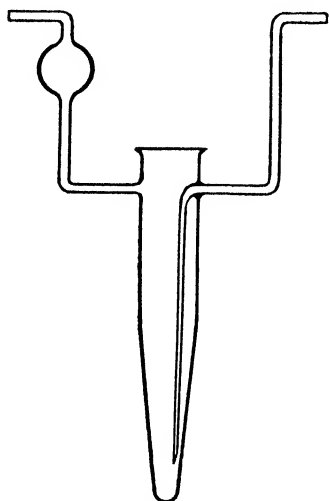
Fig. III.15.

Micro-estimation of Arsenic as Arsine.

Example 2: Micro-estimation of Arsenic after Distillation as Trichloride.*

This method is preferable if more than 50 µg. of arsenic are present.

PRINCIPLES. After wet ashing, the arsenic is reduced with ferrous salts and distilled, as arsenic trichloride, in a current of hydrochloric acid. Iodometric titration is then used to determine the arsenic in the distillate. Under *standardised* conditions of distillation antimony does not interfere.

REAGENTS.

1. *Concentrated sulphuric acid and concentrated nitric acid, arsenic-free.*

2. *Sodium hydroxide*, 20%.

3. $N/200$ *iodine solution.*

4. *Starch indicator solution*, see p. 198.

METHOD. The destruction of organic matter is carried out as described on p. 173.

After removal of nitric acid, 30 ml. of water are added to the oxidised material in the Kjeldahl flask, which is then clamped in a retort stand at

* Bang, I., *Biochem. Zeit.*, 1925, **161**, 195.

an angle and connected to a Fresenius flask which contains 30 ml. of 20% caustic soda (fig. III.16). To the Kjeldahl are added 3 g. of ferrous ammonium sulphate, and after this has dissolved, 3 g. of potassium bromide, and then 20 g. of potassium sulphate are added. The apparatus is now connected up and the Kjeldahl flask is strongly heated.

Hydrochloric acid is distilled into the alkali in the receiving flask and with it goes any arsenious chloride. Distillation is continued until hydrochloric acid ceases to be evolved. The contents of the Fresenius flask are made acid, boiled, then neutralised, and about 5 g. of sodium bicarbonate followed by 30 ml. of distilled water are added. After cooling to room temperature the solution is titrated with $N/200$ iodine solution from a micro-burette until a persistent blue occurs with starch indicator.

E. MICRO-TITRATION USING POTASSIUM BROMIDE/POTASSIUM BROMATE

The example, estimation of magnesium, illustrates the use of 8-hydroxyquinoline, "oxine," as a quantitative micro-chemical precipitant. The technique is also applicable for the micro-estimation of *aluminium, cadmium, titanium,* and *zinc.*

Organic reagents, e.g. *phenols,* can be brominated quantitatively in a similar way.
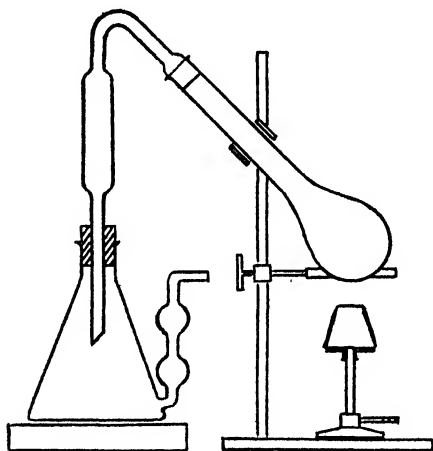
Fig. III.16. Micro-estimation of Arsenic as Arsenic Trichloride.

Example: The Micro-estimation of Magnesium after Separation as the Salt of 8-Hydroxyquinoline.*

PRINCIPLES. Magnesium is precipitated as the oxine with 8-hydroxyquinoline. The washed salt is dissolved in hydrochloric acid, and the free oxine is reacted with bromine (liberated from potassium bromide/bromate in acid solution) to give 3 : 5-dibromo-8-hydroxyquinoline.

SOLUTIONS.

1. 8-*Hydroxyquinoline, 2% solution in 2N acetic acid.* Dissolve 2 g. of oxine in 100 ml. of $2N$ acetic acid, add concentrated ammonia solution, drop by drop, till a faint cloudiness persists; warm to dissolve completely; on cooling the solution will keep clear for many months.

2. *Ammonium acetate,* 10% *solution.*

* Hasegawa, M., *Tohuku J. Exp. Med.,* 1937, **31**, 422; *Analyst,* 1937, **62**, 904.

3. *3N hydrochloric acid.*

4. *N/100 potassium bromate.* Dissolve 2·784 g. of pure dry potassium bromate in 1 l. of water and, as required, dilute portions tenfold.

5. *Potassium bromide, 10%.*

6. *Fluorescein indicator, 0·1% in 1% caustic soda.*

METHOD. Magnesium is separated from other metals in the usual manner. To the solution (about 5 ml.) contained in a 15 ml. centrifuge tube are added 5 ml. of ammonium acetate buffer and then 1 ml. of oxine solution in acetic acid. Concentrated ammonia is now added, drop by drop, with stirring until the solution is just alkaline to phenolphthalein.

The tube is then placed in a boiling water-bath for 10 minutes in order to allow a crystalline precipitate to deposit; this is then stood aside to settle. The supernatant liquid should be yellow, indicating that excess of reagent is present. The tube is then centrifuged and the supernatant is removed by suction through an upturned capillary pipette (see p. 32). The precipitate is washed twice with 3 ml. of hot water and finally is drained by inversion over filter-papers. The precipitate is dissolved in 3 ml. of 3N HCl and transferred quantitatively to a 25 ml. stoppered flask, using 3N HCl to rinse out the tube. 1 ml. of 10% potassium bromide is then added.

The titration may now be performed in either of two ways, the second of which is to be preferred.

(a) Titration Directly with Bromate, Using Fluorescein as Indicator*

To the acid solution of oxine are added a few drops of 1% fluorescein indicator and N/100 potassium bromate is run in from a micro-burette with shaking between additions. The bromate should be added slowly with swirling of the solution by rotating the flask, so as to avoid local excesses of the reagent. At the end-point, which is sharp and definite, the indicator changes from a greenish-brown to a reddish-brown.

(b) Back-titration with Thiosulphate after Addition of Excess Bromate†

To the acid solution of oxine to which bromide has been added, run in N/100 bromate from a micro-burette until an excess of bromine is present: stopper and shake thoroughly. Add 1 ml. of freshly prepared 20% potassium iodide solution and titrate the liberated iodine with N/100 sodium thiosulphate from a micro-burette using starch as indicator.

F. MICRO-TITRATION WITH POTASSIUM IODATE

The example illustrates the micro-chemical application of Andrews' iodine monochloride procedure, which depends on the reactions:

$$IO_3^- + 6H^+ + 4e = I^+ + 3H_2O : I^+ + Cl^- = ICl.$$
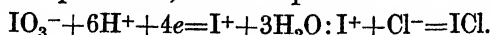
* Hahn, *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 571.
† Vogel, "Quantitative Inorganic Analysis," p. 454.

Example: The Micro-estimation of Traces of Antimony in Alloys.*

PRINCIPLES. Antimony is separated in the usual manner, reduced to the trivalent state, and titrated with potassium iodate in strong acid solution:

$$KIO_3 + 2SbCl_3 + 6HCl = KCl + 2SbCl_5 + ICl + 3H_2O.$$

SOLUTIONS.

1. *N/100 potassium iodate (M/400).* Dissolve 0·5351 g. of pure dry potassium iodate in 1 l. of water.

2. *Carbon tetrachloride.*

METHOD. About 5 g. of alloy filings are weighed into a Kjeldahl flask, 3 g. of potassium hydrogen sulphate and 10 ml. of concentrated sulphuric acid are added. The flask is strongly heated until solution is complete. After cooling, the contents of the flask are diluted to 50 ml. with distilled water and the precipitate of lead sulphate is filtered off. The filtrate is then treated with H_2S for 15 minutes and the sulphides of heavy metals are collected by centrifuging. The washed precipitates are then extracted with polysulphide in which arsenic, antimony, and tin dissolve. The solution, made acid to about $8N$ with HCl, is gently warmed. Antimony and tin sulphides dissolve, and are separated by centrifuging from arsenic and free sulphur. To the clear solution is then added about 0·1 g. of tartaric acid; an equal volume of water is added and H_2S is again passed through. Tin sulphide is allowed to collect and is centrifuged off. The clear solution containing antimony is warmed to remove H_2S, care being taken to prevent the temperature of the solution from rising to above 100° C. The solution after cooling is ready for titration.

The solution, which should have a volume of about 5 ml., is transferred to a 25 ml. ground-glass stoppered flask and 6 ml. of concentrated HCl and 0·5 ml. of carbon tetrachloride are added. $N/100$ potassium iodate is then run in from a 2 ml. micro-burette until the solution, which at first becomes pale brown, begins to fade. The flask is then shaken vigorously, when the drop of carbon tetrachloride assumes a violet colour due to free iodine. Iodate is now added, drop by drop, with shaking between each addition, until the solvent colour suddenly changes from a violet to a straw yellow. The end-point is recorded when the carbon tetrachloride layer does not regain a purple tinge within 10 minutes.

3. OXIDATION-REDUCTION TITRATIONS: ORGANIC REAGENTS

A. MICRO-TITRATION WITH DICHLOROPHENOL-INDOPHENOL

Example: The Micro-estimation of Ascorbic Acid in Blood.†

This is an example of ultra-micro titration in which an oxidation-reduction indicator is used as the titrient.

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* This is particularly useful for the estimation of antimony in commercial lead. Cf. Vogel, "Quantitative Inorganic Analysis," p. 442.

† Farmer, C., and Abt, A., *Proc. Soc. Exp. Biol. Med.*, 1936, **34**, 146.

PRINCIPLES. Ascorbic acid reduces the dyestuff: excess is shown by persistence of the colour. A micrometer syringe burette (see pp. 152–153) must be used.

SOLUTIONS.

1. *Meta-phosphoric acid*, 5%. Freshly prepared from solid stick.

2. *Dichlorophenol-indophenol* (B.D.H.). Dissolve 1 tablet, equivalent to 1 mg., of ascorbic acid in 100 ml. water. Add a drop or two of ammonia to ensure solution. Standardise each time against pure ascorbic acid.

METHOD. 0·30 ml. of blood is oxalated to prevent coagulation, and centrifuged. 0·1 ml. of the clear plasma is transferred to another centrifuge tube by means of an accurately graduated pipette, the pipette being rinsed with 0·1 ml. of distilled water which is added to the plasma. After mixing, 0·20 ml. of metaphosphoric acid is added, drop by drop, with shaking, and the tube is then centrifuged. 0·20 ml. of the clear supernatant liquid is pipetted on to a depression on a white spotting tile. In an adjacent depression are placed 0·10 ml. of water and 0·10 ml. of metaphosphoric acid. A special micrometer syringe burette, as described on pp. 152–153 (figs. III.10 and 11), is filled with dichlorophenol-indophenol solution by placing the tip
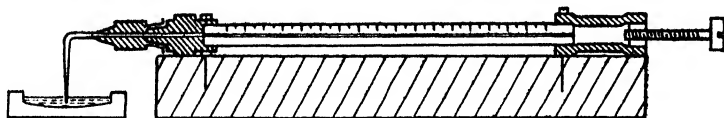
Fig. III.17.   Micrometer Syringe for Vitamin-C Titration.

below the surface of the liquid and opening the screw. (Fig. III.17 shows a simple contrivance used by the originator of this method.) The point of the capillary jet is wiped and then placed below the surface of the blood filtrate. The burette is read and the screw is gradually closed to force the dyestuff slowly out. As soon as a faint persistent pink occurs the burette is reread.

The blank solution in the adjacent depression is similarly titrated to the same depth of pink colour. The difference between blank and unknown represents dyestuff reduced by ascorbic acid in 0·06 ml. of plasma.

B. MICRO-TITRATION WITH STANDARD HYDROQUINONE

Example: The Micro-determination of Gold.*

PRINCIPLES. Gold in extreme dilution is co-precipitated with tellurium by means of sulphur dioxide. The precipitate is ignited and the residue dissolved in aqua regia. After aeration to remove nitrogen oxides, the solution is treated with *o*-dianisidine, which gives a red colour with chlorauric

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* Pollard, W., *Analyst*, 1937, **62**, 597.

acid. It is then titrated with standard hydroquinone solution, which reduces the gold to the metallic state, with consequent disappearance of the red colour.

SOLUTIONS.

1. *Standard hydroquinone solution.* Dissolve 0·4186 g. of pure hydroquinone in 200 ml. of distilled water, add 10 ml. of concentrated HCl and make up to 500 ml. (1 ml. ≡ 1 mg. Au).

2. *Standard gold solution.* Dissolve 0·50 g. of gold in 2 ml. of nitric acid and 6 ml. of HCl. Remove dissolved gases by passing a current of air through the solution for 5 minutes, then make up to 500 ml. with distilled water.

3. *o-Dianisidine indicator.* Dissolve 0·5 g. of recrystallised o-dianisidine in 200 ml. of water, add 2 ml. of concentrated HCl, and make up to 500 ml. with distilled water.

4. *Tellurium solution.* Dissolve 5 g. of metal in 20 ml. of HCl and 5 ml. of HNO_3; evaporate to a thick syrup and remove all HNO_3. Add 25 ml. of concentrated HCl and dilute to 500 ml. Each millilitre of solution contains 100 mg. tellurium.

5. *Acid potassium fluoride buffer.* 5% solution of KHF_2.

METHOD. If the sample is an ore or an alloy it is parted by action with nitric acid. The resulting gold is then dissolved in bromine water and aerated to remove excess of bromine. If the gold is in solution it is made about N acid with concentrated HCl.

2 ml. of tellurium solution are added to each litre of solution. Sulphur dioxide is then passed in until saturation, and the solution is allowed to stand over-night. The tellurium is precipitated in granular form and carries down with it any gold. The precipitate is filtered on a Buchner funnel. The filter-paper and precipitate are transferred to a small porcelain crucible, dried, and then ignited. In this way the bulk of the tellurium is volatilised, but a little is left as the dioxide. After cooling, the residue in the crucible is treated with 6 drops of concentrated HCl and 2 drops of concentrated HNO_3. The crucible is then placed on a water-bath for 15 minutes to dissolve the precipitated gold, and then in a corked tube fitted with a piece of glass tubing which almost touches the liquid, so that air may be drawn over its surface. This process is carried out for 5 minutes, wherein all nitrogen oxides and free chlorine are removed. More vigorous treatment is to be avoided, since it tends to cause chlorauric acid to lose chlorine.

To the solution in the crucible are then added 1 ml. of acid potassium fluoride and 1 ml. of indicator. Immediately, standard hydroquinone is run in from a micro-burette until the red colour is just discharged. The end-point is quite sharp and is best seen against a white background.

This method is capable of recovering one part of gold in a thousand million parts of solution, and if more than 2 mg. of gold are present an aliquot containing less than this amount should be taken.

4. MICRO-CHEMICAL ESTIMATION OF CHLORIDES

· Since the micro-chemical estimation of chloride ions is an analysis in frequent demand, the alternative analytical methods have been exemplified in such a way as to show how typical practical difficulties may be overcome.

In making the choice of a suitable procedure the following considerations should be taken into account.

1. **The Volhard procedure (i.e. thiocyanate titration)** is used most generally, and is convenient for estimating quantities of chloride down to 0·1 mg., but its weakness lies in the gradual fading of the end-point.

2. **The mercuric nitrate procedure** is more sensitive, and is suitable for quantities of the order of 10–100 μg. It cannot be used in the presence of other heavy metals or of substances which form mercuric complexes, such as ammonium salts, organic bases (e.g. purines in urine), or sulphur compounds. The pH range for accurate work is very critical (see p. 182).

3. **The mercuric oxy-cyanide method** (pp. 87–88), though very sensitive and neat, has a still more limited applicability. It is not very accurate with buffered solutions.

4. **The silver iodate procedure** (p. 183) illustrates ionic interchange between sparingly soluble salts. The eventual iodide titration is very sharp and well adapted to ultra-micro work with quantities of the order of 10 μg. of chloride.

5. **The absorption indicator method** is more suitable to semi-micro work (0·5–10 mg.) and has the advantage of rapidity. Theoretical considerations have been discussed on pp. 140–141.

6. **Mohr's method (use of chromate indicator)** is advocated for micro-chemical work by Bang (see p. 197), and by following his exact technique can be used for quantities of 0·05–0·5 mg. The titration must be carried out in neutral *unbuffered* solution.

A. MICRO-TITRATION WITH STANDARD THIOCYANATE (VOLHARD PROCEDURE)

Example: · The Estimation of Chlorine in Whole Blood.*

PRINCIPLES. Organic matter is destroyed by heating with nitric acid and persulphate in the presence of excess of silver nitrate, which precipitates

*\,Smirk, F., *Biochem. J.*, 1927, **21**, 31.

the chloride. Back-titration is with thiocyanate, using ferric alum as indicator. Acetone must be added to prevent occurrence of the reaction:

$$AgCl \text{ (solid)} + NH_4CNS \longrightarrow AgCNS \text{ (solid)} + NH_4Cl.$$
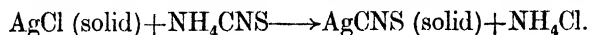
REAGENTS.

1. *N/50 silver nitrate.*
2. *N/50 ammonium thiocyanate.*
3. *Sodium persulphate, solid.*
4. *Ferric alum, a saturated solution.*
5. *Concentrated nitric acid*, chloride-free.
6. *Acetone, A.R.*
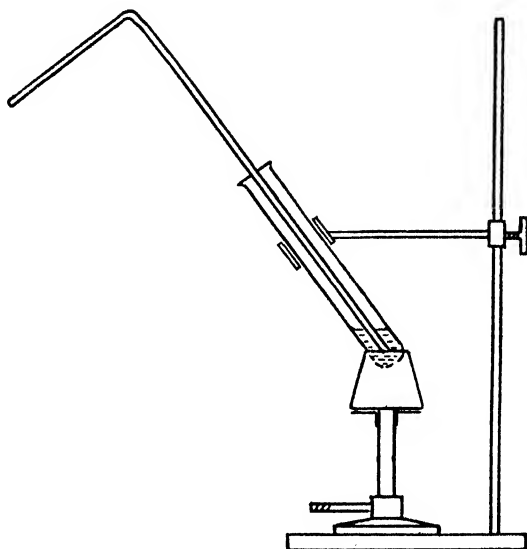
Fig. III.18. Destruction of Organic Matter with Nitric Acid.

METHOD. 0·20 ml. of blood is pipetted into the bottom of a Pyrex test-tube (6 in. × ¾ in.) and the pipette is rinsed with distilled water into the tube. 2·00 ml. of N/50 silver nitrate are then added, followed by approximately 0·3 g. of solid persulphate, which is washed down into the tube with 3 ml. of concentrated nitric acid. The contents are then heated gently until the protein has dissolved.

The tube is clamped in a retort stand at an angle of about 30° from the vertical and air is bubbled through the liquid by means of a fine capillary (see fig. III.18). This prevents bumping and assists oxidation. The contents are boiled freely for about 30 seconds when the original opalescence due to colloidal silver chloride vanishes and there remains a clear, greenish solution containing the chloride precipitate in small clumps. The capillary

is removed, rinsed down with a few drops of distilled water, and the tube is set aside to cool.

0·5 ml. of saturated ferric alum solution is added, followed by 10 ml. of acetone. The solution is now titrated with $N/50$ thiocyanate from a 2 ml. micro-burette fitted with a capillary tip. The end-point, which is quite sharp, is indicated by the appearance of a distinct pink colour, which persists for 30 seconds after mixing, since the back reaction due to the solubility of silver chloride is much repressed by the addition of acetone.

B. MICRO-TITRATION OF CHLORIDE USING MERCURIC NITRATE*

PRINCIPLES. If standard mercuric nitrate be added to a solution of a chloride in acid, the mercuric chloride which forms is only very slightly dissociated. Mercuric ions occur in solution when excess of mercuric nitrate has been added, and this is indicated by means of the bluish colour which results from the formation of a complex of mercuric ions and diphenylcarbazide.

SOLUTIONS.

1. $N/250$ *mercuric nitrate.* Dissolve 2·166 g. of mercuric oxide in 30 ml. of N HNO_3; make up to 1 l. with water. This stock solution is $N/50$ with respect to mercury and $N/100$ to nitric acid. Mix 200 ml. of this solution with 8 ml. of N HNO_3 and dilute to 1 l. with water. This dilute solution is $N/250$ to Hg and $N/100$ to HNO_3.

2. N *nitric acid.*

3. *Diphenylcarbazide.* 0·1% solution in methyl alcohol.

4. 2 : 5-*Dinitrophenol.* 0·1 g. dissolved in 20 ml. of alcohol and diluted to 100 ml. with water.

METHOD. The sample should be contained in about 3 ml. of solution: proteins and substances which form complexes with mercuric salts in acid solution should be absent. The solution is neutralised with dilute caustic soda, a few drops of dinitrophenol indicator are added, and dilute nitric acid is run in until the yellow colour is just discharged. The solution is now brought to pH 2 (i.e. $N/100$ with respect to nitric acid) by adding the calculated amount of N HNO_3. (If buffers are present, this must be determined by pH measurement.)

For every millilitre of solution is now added 0·1 ml. of indicator. $N/250$ mercuric nitrate is now run in slowly from a micro-burette until there appears a permanent violet colour when the mixture is viewed against a white background in the light from a daylight lamp. The end-point can be determined to 0·005 ml. in a total bulk of under 5 ml., and 10 μg. of chloride may be estimated to an accuracy of within 10%, 100 μg. to within 1%.

* Roberts, I., *Ind. Eng. Chem.* (Anal. Edn.), 1936, **8**, 365; Moody, F. C.: Private communication.

C. Micro-titration using Silver Iodate

Example: Micro-determination of Chloride in Biological Fluids.*

PRINCIPLES. When a soluble chloride is shaken with silver iodate powder, insoluble silver chloride precipitates and soluble iodate appears in stoichiometric proportion.

A filtrate is then treated with acid potassium iodide and the liberated iodine is titrated with thiosulphate.

SOLUTIONS.

1. $0 \cdot 085M$ *phosphoric acid.*

2. *Capryl alcohol.*

3. *Potassium iodide,* 5%, freshly prepared.

4. *Silver iodate.* Powder, A.R.

5. *Starch solution,* 2%. See p. 198.

6. $N/250$ *sodium thiosulphate.* Dissolve 30 g. of sodium thiosulphate in 1 l. of water. Allow to age for 1 week in a dark bottle before standardising. Dilute this solution 40–50 times immediately before use.

7. *Standard potassium bi-iodate solution.* A $0 \cdot 05M$ solution is made by dissolving 19·498 g. of $KH(IO_3)_2$ in 1 l. of water. For titration, this stock solution is diluted 200 times with $0 \cdot 085M$ phosphoric acid solution.

METHOD. 0·020 ml. of plasma is delivered from a pipette, calibrated *to contain,* into a small Pyrex test-tube into which was previously placed 0·6 ml. of $0 \cdot 085M$ H_3PO_4 solution. A thin glass rod dipped in capryl alcohol and shaken free of the adhering drop is touched on the surface of the liquid. 6 mg. of solid silver iodate are now added and the tube is covered with a rubber cap and shaken vigorously for 2 minutes. The tube is then centrifuged for 1 minute. 0·50 ml. of clear supernatant liquid is withdrawn, with a pipette calibrated between marks, using a rubber teat as a suction device, and transferred to a smaller titration tube. 1·0 ml. of $0 \cdot 085M$ H_3PO_4 and 0·1 ml. of 5% potassium iodide solution are added and the tube is allowed to stand for 5 minutes. The dilute standard thiosulphate is run in from a micro-burette until the yellow colour is just perceptible. One drop of starch solution is then added and the titration is continued until the blue colour is completely discharged.

Since silver iodate has a slight solubility, a blank allowance must be made for this and for any chloride impurity which might be present in the phosphoric acid used. This is obtained by taking 0·50 ml. of the acid, adding 6 mg. of the solid $AgIO_3$, and subjecting 0·5 ml. of the supernatant fluid, after shaking and centrifuging, to the procedure given above.

For routine work a series of standard chloride solutions should be estimated as described above: from the results should then be constructed a calibration curve relating thiosulphate titre to chloride in solution.

* Sandroy, *J. Biol. Chem.,* 1937, **120**, 335–417.

D. MICRO-TITRATION OF CHLORIDES USING AN ADSORPTION INDICATOR*

Example: Estimation of Chlorides in Biological Fluids.*

PRINCIPLES. Chloride is extracted from blood, etc., with concomitant precipitation of proteins, by the use of alcohol-ether mixture. The clear filtrate is titrated with silver nitrate in the presence of dichlorofluorescein indicator. As soon as free silver ions are present in the solution, the silver dichlorofluoresceinate which forms is strongly adsorbed on to the silver chloride precipitate, which then undergoes a sharp colour change from yellow to red.

SOLUTIONS.

1. 3 *parts of absolute alcohol to* 1 *part of anhydrous ether.*
2. *Dichlorofluorescein,* 0·05%, *in* 75% *alcohol.*
3. *N*/50 *silver nitrate.*

METHOD. Pipette 0·50 ml. of oxalated plasma into a 50 ml. Pyrex centrifuge tube, containing 25 ml. of alcohol-ether; stopper and shake thoroughly and then centrifuge at 2,000 r.p.m. for 5 minutes. 20 ml. of the clear supernatant fluid, or filtrate, are pipetted into a 50 ml. Erlenmeyer flask, 0·4 ml. of dichlorofluorescein indicator is added, and the solution is titrated with *N*/50 silver nitrate from a micro-burette of the horizontal type (see p. 149). The flask should be shaken during the titration, and as the end-point is approached vigorous shaking between each addition of silver nitrate is essential. The end-point is characterised by a sharp colour change. The solution, which initially exhibits a yellowish colour with a greenish fluorescence, changes to a distinct reddish tinge. On further shaking the precipitate becomes a red coagulum with the indicator completely absorbed from the solution.

5. SPECIAL INORGANIC TITRATION PROCEDURES

A. ESTIMATION OF TRACES OF WATER BY KARL FISCHER'S METHOD†

The determination of small quantities of free water in oils or powdered solids often presents difficulty, since the standard Dean and Stark method of distillation with an inert solvent is incapable of detecting less than 0·1 ml. of water.

Karl Fischer's procedure will easily detect 1 mg. of water, and there need be no limit to the size of the sample taken. It is, of course, inapplicable with substances which readily absorb iodine (e.g. unsaturated oils), but this difficulty can often be overcome by a preliminary treatment with iodine in dry carbon tetrachloride.

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* Saifer, H., Hughes, J., and Weiss, E., *J. Biol. Chem.*, 1942, **146**, 527.
† Karl Fischer, *Angew. Chem.*, 1935, **48**, 394; Smith, D., Bryant, W., and Mitchell, J., *J.A.C.S.*, 1939, **61**, 2407.

Several substances which yield water by chemical reactions (e.g. esterifications) can also be estimated by means of this reagent.

The method becomes particularly sensitive if the end-point is determined electrometrically, as described on p. 431.*

PRINCIPLES. Pyridine, iodine, and sulphur dioxide together react quantitatively with water to produce pyridinium salts, the iodine being reduced. The substance to be analysed is thoroughly shaken with water-free methyl alcohol and titrated with the reagent until excess is present as shown by the colour of free iodine.

The reactions are probably as follows:
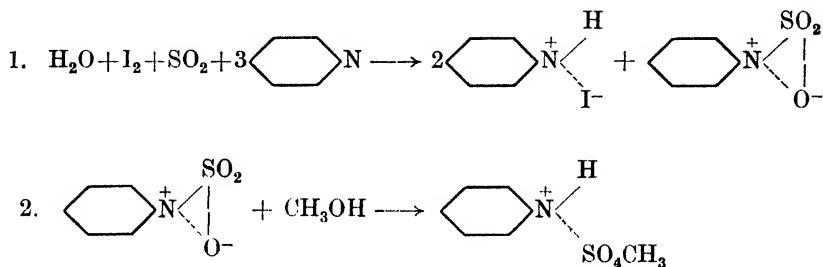
REAGENT. Dissolve 84·7 g. (0·33M) of pure iodine in 269 ml. (3·3M) of pyridine and 667 ml. of pure *dry* methyl alcohol. Cool in chopped ice and add 64 g. (1M) of liquid SO_2 cautiously. The reagent is then ready for use. It is kept in a dark automatic-filling burette fitted with a calcium chloride guard tube. (The water content of the solvent should not be greater than 0·1%, since 6 g. of water will exhaust 1 l. of reagent.) The reagent deteriorates rapidly for a week and then more slowly. Hence it is convenient to make up the mixture and add the SO_2 as required.

The reagent must be standardised immediately prior to use. This is done as follows: weigh out about 18 ml. of water into a flask and dilute to 1 l. with pure dry methyl alcohol. Titrate 10 ml. portions of this solution with the reagent until the colour of the iodine persists.

METHOD. The sample containing water is weighed into a ground-stoppered Erlenmeyer flask, to which is then added about 25 ml. of water-free methyl alcohol or dioxane. The flask is stoppered and thoroughly shaken to extract the water into the solvent. The solvent is then titrated from the burette with the Karl Fischer reagent. This is added slowly, with shaking, and as the reaction takes place so the colour changes from brown to pale yellow. The end-point is reached when a persistent brown colour due to free iodine is observed.

* Levy, Murtaugh, and Rosenblatt, *Ind. Eng. Chem.* (Anal. Edn.), 1945, **17**, 193,

B. Micro-titration Using Tetrahydroxy-Quinone, "THQ," as Indicator

Example: Micro-estimation of Sulphate.*

PRINCIPLES. Tetrahydroxy-quinone forms a red lake with barium salts. Sulphate solutions at pH 7 may therefore be titrated with standard barium chloride, excess of which forms the distinctly coloured lake. 100 µg. of sulphate can be estimated in this way.

Substances which form insoluble barium salts in neutral or slightly alkaline solution interfere with the titration, but often these can be eliminated by pre-treatment with lime-water. Neutral salts in high concentration, especially chlorides, modify the end-point.

SOLUTIONS.

1. *N*/100 *barium chloride.*
2. *N*/50 *hydrochloric acid.*
3. *N*/50 *caustic soda.*
4. *Ethyl alcohol, 95%.*
5. *Tetrahydroxy-quinone* (solid).

METHOD. The substance containing sulphate (1–5 mg.) is brought into solution in about 20 ml. of liquid, adjusted to pH 7 with acid and alkali (just colourless to phenolphthalein), and 25 ml. of ethyl alcohol are added. 0·15 g. of "THQ" indicator is added and the liquid is titrated with N/100 barium chloride from a micro-burette. Shaking must be made between additions, and the end-point is determined by matching against a like tube containing 50 ml. of 50% alcohol, 0·20 ml. of barium chloride, and 0·15 g. of THQ.

At the end-point the colour of the solution changes from yellow to a reddish tinge.

The operations must be standardised by comparison with known solutions of sulphate containing similar amounts of sodium chloride, and a graph should be constructed relating titration with sulphate concentration. Only by following rigidly the conditions under which the calibration curve was made may reproducible results be obtained.

C. Micro-titration of Zinc with Potassium Ferrocyanide†

PRINCIPLES. o-Dianisidine is a sensitive oxidation-reduction indicator for ferrocyanide-ferricyanide mixtures, and may be used to mark the end-point of precipitation reactions of standard potassium ferrocyanide.

Zinc is titrated in acid solution with $K_4Fe(CN)_6$: $Zn_2Fe(CN)_6$ is precipitated; excess of soluble ferrocyanide lowers the oxidation potential

* Hallet, H. L., and Kuipers, J., *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 360.
† Frost, H., *Analyst*, 1943, **68**, 51.

of the system, initially containing a little ferricyanide, and this produces a colour change with the o-dianisidine.

Other oxidation-reduction indicators which may be used are diphenyl-amine sulphonic acid and diphenylbenzidine, but these do not give such sharp end-points.

SOLUTIONS.

1. *Indicator.* 0·1 g. of o-dianisidine in 0·5 g. of concentrated sulphuric acid diluted to 100 ml. with water.

2. *Potassium ferricyanide, 5%, in water.*

3. *N/20 potassium ferrocyanide.*

METHOD. The sample containing zinc is suitably dissolved and the zinc is separated from other metals in the usual manner. The zinc is finally brought into solution in about 20–30 ml. of water, 10 g. of ammonium chloride are added and then 5 ml. of concentrated HCl. The mixture is heated to boiling and then a few drops of the indicator are added. After titration with $N/20$ ferrocyanide has been commenced from a micro-burette, a few drops of ferricyanide are added to produce a reddish-brown colour.

The titration is continued with the liquid at boiling temperature until the colour of the indicator changes to a pale bluish-green. A very sharp end-point may be obtained, particularly if trial titrations are made with standard zinc solutions to familiarise the technician with the colour change.

D. MICRO-TITRATION OF VANADATES WITH LEAD NITRATE, USING A DIPHENYL-CARBAZIDE INDICATOR*

PRINCIPLES. A reagent prepared by adding diphenyl carbazide to pyridine and nitric acid gives an intense colour with lead and also with vanadium. Vanadate solutions when titrated with standard lead nitrate in the presence of this indicator show a decreasing colour as the end-point approaches, since lead vanadate is insoluble, and then a sudden return of colour as excess of lead is added.

The principle may be used for the estimation of zinc also. *Tungstates, molybdates, phosphates,* and *arsenates* can also be titrated with standard lead solutions by this method, but in these cases the colour appears only when excess of lead is present in the solution.

SOLUTIONS.

1. *Indicator.* Mix 30 ml. of purified pyridine and 120 ml. of distilled water with 2 ml. of nitric acid (sp. gr.=1·2); add 10 ml. of a 1·5% solution of diphenyl-carbazide in alcohol and allow the mixture to stand over-night.

2. *Lead nitrate solution.* 1 g. of $Pb(NO_3)_2$ in 1 l. of water.

3. *Pure acetone.*

* Evans, B. S., *Analyst*, 1939, **64**, 1.

METHOD. The vanadate, contained in 10 ml. of solution, is neutralised with dilute ammonia and dilute HCl.   1 ml. of the reagent is added and then 3 ml. of pure acetone.   The standard lead solution is added from a micro-burette slowly, with shaking between additions.   The reddish colour which appears on addition of the reagent gradually fades as the lead is added and lead vanadate is precipitated, until only the colour of the reagent is left.   Another drop of standard solution will then produce a red colour due to the lead complex.

The titration is best carried out against a comparison tube containing the same volume of liquid, to which has been added one drop of lead solution. Allowance should be made for this excess when calculating the result.

This titration is especially suitable and sensitive for micro quantities of vanadium; quantities above 1 mg. tend to give low results and should be diluted accordingly.

1 mg. of vanadium is equivalent to 8·12 ml. of lead nitrate solution, and a titration may be made to 2 μg. with ease and reproducibility.   Carbonates must be excluded, since in neutral solution these titrate as do the other anions mentioned above.

E. MICRO-TITRATION OF METALS USING "DITHIZONE"

As mentioned in Part I (pp. 45–47), many metals form dithizone com-plexes soluble in organic solvents such as chloroform and carbon tetrachloride.

The reaction may be made specific for certain metals by altering the conditions of reaction, e.g. by adjusting the pH of the solution to be titrated,* and may be developed both as a volumetric and as a colorimetric procedure of high accuracy.   Pd, Au, Hg^{++}, and Cu^{++} can be separated from other metals at pH 1·0, whilst Pb and Bi can be separated at pH 3.   The dithizonates of other metals form at higher pH's, and can be separated by complex salt formation with cyanide, thiocyanate, or thiosulphate in solutions buffered with citrates or tartrates.

The example given below—estimation of traces of lead—illustrates the technique of the volumetric procedure and shows its extreme sensitivity.

Particular care must be paid to the purification of the dithizone reagent, oxidation of which should be prevented by use of hydroxylamine.   All other solutions must be free from even minute traces of heavy metals. Water distilled from all-glass apparatus should be used to prevent con-tamination by traces of copper.

Example: Estimation of Lead Dust in Atmospheres.†

PRINCIPLES. Lead is suitably obtained in solution.   It is shaken with a chloroform solution of dithizone, when the complex passes into the

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* Cf. Sandell, "Colorimetric Determination of Traces of Metals" (Interscience Publishers, New York, 1944).

† Horwitt, M., and Cowgill, G., *J. Biol. Chem.*, 1937, **119**, 553.

non-aqueous layer. Excess dithizone is removed and then the lead is extracted with acid. The dithizone equivalent to the lead is then titrated with a standard lead solution.

SOLUTIONS.

1. *Dithizone solution*. Dithizone must first be purified. Dissolve 1 g. in 75 ml. of chloroform, filter if necessary, shake with four 100 ml. portions of metal-free distilled 1% ammonia solution, into which the dithizone passes. Unite the aqueous layers and filter from chloroform through a cotton-wool plug. Acidify with dilute hydrochloric acid and extract the precipitated dithizone with two or three 20 ml. portions of chloroform. Wash with water and strip off the bulk of the chloroform, transfer to a beaker and take down to dryness by heating for about 1 hour at 50° C. *in vacuo*. Dissolve about 40 mg. of this dithizone in 400 ml. of chloroform and filter into a separating funnel. Add 50 ml. of water containing 2 ml. of 25% hydroxylamine hydrochloride and shake.

Keep in a cool dark place and use as required. The acid aqueous layer prevents oxidation and extracts any lead, etc., from the reagent.

2. *Potassium cyanide, 0·5% solution, lead-free*. Place 100 ml. of 10% cyanide in a separating funnel. Extract with 2 ml. of chloroform containing 2 drops of dithizone solution. If a pink colour appears, remove it and extract with further dithizone solution till this no longer indicates lead. Dilute to 0·5% as required.

3. *Standard lead solution*. Dissolve 1·599 g. of lead nitrate in 1 l. of 0·1% nitric acid: the solution contains 1 mg. of Pb per millilitre. When required dilute 10 ml. of this stock to 1 l. to give a solution containing 0·01 mg. per millilitre.

4. *Sodium citrate solution*. Mix 500 ml. of 20% sodium citrate solution with 5 ml. of 10% potassium cyanide solution and extract with 10 ml. portions of dithizone solution in chloroform until lead-free. Acidify with 2·5 ml. of 20% HCl and extract excess dithizone with chloroform.

TECHNIQUE FOR SAMPLING LEAD DUST IN ATMOSPHERES*

The particulate filter shown in fig. III.19 is connected with a flowmeter and a suction device, and a given volume of air is drawn through to give a reasonable sample (not less than 0·05 mg. of lead). The dust is extracted from the filter with small quantities of nitric acid, and the mixture is evaporated to dryness in a beaker. The residue is taken up with 15 ml. of 50% HCl, warmed, then transferred to a 100 ml. volumetric flask and finally is made up to the mark with distilled water, after adding 1 ml. of 25% hydroxylamine hydrochloride and adjusting to *p*H 8 with dilute ammonia.

* This technique should be used generally for dust sampling, e.g. for collecting As, Cd, Zn, etc., in flue gases.

ANALYTICAL METHOD. Take an aliquot containing about 0·05 mg. of lead, dilute to 75 ml. with water, and add, drop by drop with shaking, 0·5 ml. of 10% potassium cyanide solution. Then add 0·5 ml. of dithizone solution and 4 ml. of chloroform. Shake and separate. If the chloroform layer does not show excess of dithizone, then re-extract with further portions of 0·2 ml. of dithizone reagent and 2 ml. of chloroform until such is the case. To the combined chloroform solution add 1·5 times its volume of 0·5% cyanide solution, shake for 10 seconds, separate, and wash the aqueous layer with 1 ml. of chloroform. Combine the chloroform solutions and again extract with 1·5 times its volume of 0·5% cyanide. Wash the aqueous layer with 2 ml. of chloroform and combine the chloroform solutions. In this way the lead complex is completely separated from the excess of dithizone.

Fig. III.19.
Particulate Filter for Dust Analysis.

The two portions of the metal, or bake-lite, box screw together and grip a disc of hardened filter-paper of adequate porosity. When a sufficient volume of smoke, etc., has been passed through the filter, the box is taken apart and the filter pad is removed as a whole for extraction. The exit gases from the particulate filter should be passed through a flowmeter or other volume-recording instrument.

The chloroform solution is then shaken for 15 seconds with 2 volumes of 0·5% hydrochloric acid. The lead passes into the acid layer and the equivalent of free dithizone is left in the chloroform solution. The aqueous layer is extracted with 1 ml. of chloroform and the solvent extracts are combined. To the chloroform solution is then added half its volume of 0·5% cyanide solution. The bulk of the dithizone then passes into the aqueous layer, which then assumes a brown colour. This is titrated from a 5 ml. micro-burette with standard lead solution, drop by drop, with shaking between additions, until the aqueous layer is almost colourless, indicating that the dithizone-lead complex has passed into the chloroform phase. This layer is then removed and the aqueous fraction is washed with 2 ml. lots of chloroform until the layer is colourless. Lead solution is then added again, drop by drop with shaking, and the chloroform layer, which becomes red, is removed. More chloroform (2 ml.) is then added and the titration is continued until the chloroform no longer extracts any red colour. At the actual end-point there is a slight pink in the chloroform solution, the addition of one further drop to the separated solution causing no colour in a fresh chloroform addition.

Best accuracy in the titration is attained if there is set up a comparison

tube containing 2 ml. of 0·5% cyanide solution, 2 ml. of chloroform, and 1 drop of dithizone to which has been added 1 measured drop of the lead solution. This gives a faint pink in the chloroform layer equivalent to about 0·5 µg. of lead. The unknown is titrated to this colour and allowance is made in the computation.

F. Micro-titration of Fluorides with Thorium Nitrate

The estimation of fluorides is a reaction of importance. It depends on the formation of a complex fluoride of a heavy metal (Th), which is thereby prevented from forming a lake with a mordant dyestuff. Aluminium, beryllium, zirconium, and certain of the rare earths can also be used in titrations of this type, and conversely aqueous fluorides can be used for the estimation of these metals.

The initial separation of fluorine by distillation is a procedure which requires careful control and a blank test must be run with both apparatus and reagents.

Volatile organic compounds of fluorine should first be decomposed in a Parr bomb (p. 82).

Example: The Determination of Fluorine in Foods.*

PRINCIPLES. Organic matter is destroyed by ashing in the presence of lime. Fluorine is removed as hydrofluorosilicic acid by distillation in the presence of excess of perchloric acid. The distillate is titrated with a standard thorium solution in parallel with a control solution of known fluoride content. At the end-point thorium–alizarin–S lake is bleached.

REAGENTS.

1. *Fluorine-free lime.* Prepare an ammonium carbonate solution by dissolving 110 g. of A.R. ammonium carbonate and 55 ml. of ammonia (sp. gr. 0·880) in water and diluting to 600 ml. Dissolve 200 g. of pure calcium chloride in 600 ml. of distilled water. Add 20 ml. of ammonium carbonate solution; stir, bring to boil, allow to settle, filter off on a Buchner funnel and reject the precipitate. Repeat the precipitation and filtration three times with 20 ml. portions of the carbonate reagent. Treat the clear filtrate after the last precipitation with the remainder of the carbonate reagent, stir well, bring to the boil, allow to settle, filter, wash several times with hot water until free from chlorides. Finally, dry at 100° C. and ignite in a platinum dish in 1 or 2 g. quantities as required.

2. *Silver sulphate.*

3. *Perchloric acid,* 60%.

4. *N/20 sodium hydroxide.*

5. *N/20 hydrochloric acid.*

6. *Alizarin–S* (or *Solochrome Blue*), 0·01% solution in water.

* Official method of the Society of Public Analysts: *Analyst,* 1944, **69**, 243.

7. *Thorium nitrate,* 0·024% *solution.*

8. *Standard solution of sodium fluoride.* 0·0221 g. of NaF per litre (1 ml. ≡ 10 mg. of fluorine).

APPARATUS. The distillation apparatus consists of a 100–150 ml. Claisen flask. The main neck of the flask is fitted with a two-holed rubber stopper through which pass a thermometer and a glass tube connecting with a steam supply—both of which project almost to the bottom of the flask. The side-neck of the flask is closed with a rubber stopper and the side-arm connects with a condenser. The steam is generated from water made alkaline with caustic soda. Local overheating of the Claisen flask is avoided
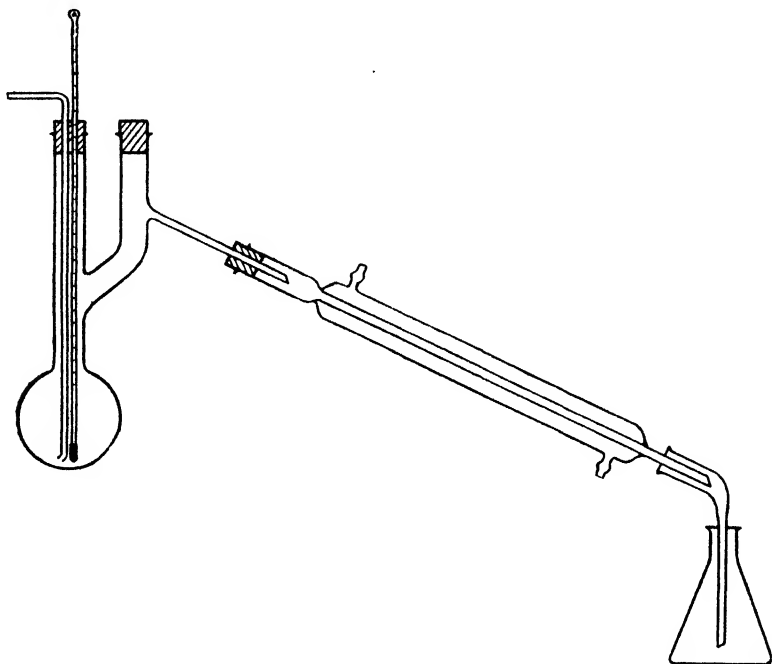
Fig. III.20. Distillation of Silicon Fluoride.

by use of an asbestos board with a hole which must fit closely to the bottom of the flask.

METHOD.

(i) *Distillation.* About 10 g. of the sample are mixed in a platinum dish with about 1 g. of fluoride-free lime, evaporated on a water-bath, charred at below red heat, transferred to a muffle at dull red heat (about 600° C.), and then ignited for 2 hours.

In the Claisen flask (fig. III.20) are placed a few fragments of Pyrex glass, sufficient silver sulphate to precipitate all the chloride in the sample, 7 ml. of water, and 15 ml. of perchloric acid. The flask is heated until the

temperature is 120°–125° C., connected to the steam supply, and regulated so that the distillation occurs at 137°–140° C.   150 ml. of liquid are distilled over in about 25–35 minutes, and the condenser is steamed out at the end of the distillation.   This distillate, which contains the bulk of the fluoride impurities in the reagents and apparatus, is discarded.   A further 150 ml. are distilled and titrated as described below.   This figure gives the residual blank and should not exceed 1·5 μg. of fluorine.

The flask is cooled, rinsed with distilled water, and the acid is transferred to a steamed-out receptacle.   The bulk of the dry ash (above) is tipped into the flask and the remainder washed in, using a few drops of the distilled acid mixture and about 5 ml. of water.   The remainder of the acid is then added, and, while cooling, the sides of the flask are washed down with a few millilitres of water.   The apparatus is connected up and 150 ml. are distilled as before.

(ii) *Titration.* 50 ml. of the distillate are titrated with $N/20$ NaOH in a Nessler tube until the colour to methyl orange is identical with that of a comparator tube containing distilled water and the indicator.

The remaining 100 ml. of distillate are transferred to another Nessler tube and the calculated amount of $N/20$ HCl to make the solution contain the equivalent of 5 ml. of $N/20$ acid is added.   A control cylinder containing 100 ml. of distilled water and 5 ml. of $N/20$ HCl is prepared.

To both cylinders 2 ml. of 0·01% alizarin–S solution are added.   Thorium nitrate solution is added from a micro-burette, graduated to 0·01 ml., to the test cylinder until a faint pink colour persists, as compared with the yellow colour of the control.   An *exactly* similar volume of thorium nitrate is added to the control cylinder, which will then become a deep pink.   Then the control solution is titrated from a micro-burette with a standard solution of sodium fluoride until the colours in the two tubes match exactly.

The volume of standard fluoride solution used corresponds to the amount of fluorine in the test portion (100 ml.) of the distillate.   The fluorine content of the analysed sample is that of the full distillate (150 ml.) less the apparatus blank.

If *Solochrome Blue* is used as indicator in place of alizarin–S the colour change is much more sensitive.*

G. Micro-estimation of Potassium by Methylene Blue Titration
    after Precipitation as Picrate†

PRINCIPLES.   Potassium is precipitated as an insoluble picrate.   The washed precipitate is dissolved in hot water and titrated with standard methylene blue.   The equilibrium

$$K \; picrate + Methylene \; blue \; (chloride) \rightleftharpoons KCl + M \; blue \; picrate$$

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* Milton, R., Liddell, H., and Chivers, J., *Analyst*, 1947, **72**, 43.
† Bolliger, A., *J. Biol. Chem.*, 1934, **107**, 229.

is displaced by the removal of methylene blue picrate from the aqueous phase by extraction with chloroform, and so the double decomposition reaction proceeds to completion. Thus the aqueous layer does not become blue until an excess of dye has been added.

This extraction technique is a novel procedure, but is, however, illustrative of a general theoretical principle.

SOLUTIONS.

1. *Calcium picrate in alcohol*, 25%. Boil 24 g. of picric acid and 10 g. of calcium carbonate in 100 ml. of water until all evolution of carbon dioxide ceases. Filter the solution and evaporate to dryness. Dissolve the residue in 100 ml. of alcohol and filter.

2. $N/100$ *methylene blue solution*. Dissolve 3·74 g. of chemically pure methylene blue (Merck) in 1 l. of water.

3. *Purified ether containing* 4% *ethyl alcohol.*

METHOD.

(i) *Picrate separation.* The sample should be freed from ammonium salts and the potassium should be present as the chloride or perchlorate. In the case of biological material, this is carried out by using the oxidation technique described on p. 101, wherein all ammonium salts are eventually removed by sublimation. Calcium and magnesium do not interfere, but if sodium is present in great excess over potassium it is best to separate the latter as perchlorate as a preliminary.

To the sample, contained in about 1 ml. of liquid, about 20–30 volumes of calcium picrate solution are added. The container is stoppered, thoroughly shaken, and allowed to stand over-night at room temperature. The mixture is then filtered, using the filter-stick technique (p. 21) and the precipitate and flask are washed three times with 4 ml. of 4% ethyl alcohol in purified ether. To the flask are added 5 ml. of pure ether, and the filter-stick is dipped therein and allowed to stand for 5 minutes. If at the end of this period the ether is colourless, then it is sucked through the stick and the precipitate is ready for titration. In the presence of a concentration of sodium of the same order as potassium, sodium picrate also partially precipitates and must be washed into solution with the ethyl alcohol/ether mixture. A yellow tinge in the ether solution after standing in contact with the precipitate denotes that this removal by washing is incomplete and the procedure must be extended until the ether is colourless.

(ii) *Titration.* To the flask are now added 5 ml. of hot water. The filter-stick is then fitted with a rubber teat and the water is forced in and out of the filter to dissolve all the precipitate. After cooling, 5 ml. of chloroform are added to the flask and the solution is titrated with standard methylene blue solution. The flask is stoppered and shaken between the additions of the methylene blue.

Methylene blue picrate passes into the chloroform layer and colours it green. The end-point is reached when the aqueous solution becomes colourless. The addition of a further drop of the methylene blue solution should cause the aqueous layer to become permanently blue. The methylene blue solution must be standardised against a known solution of picric acid titrated in the above manner.

The method is particularly suitable for up to 3 mg. of potassium, and is recommended for estimation of potassium in fruits, etc.

SPECIAL BIOLOGICAL PROCEDURES

A. BANG'S METHODS AND PRINCIPLES

Bang's system of blood analysis was elaborated so that, using only small quantities of blood, the minute amounts of the various circulating metabolites could readily be estimated. The system was so framed that a number of estimations for different substances could be made from the same sample, and was of such simplicity as to allow of mass analysis.

The procedure (essentially micro in that not more than 100 mg. of blood are used) was built round the use of the torsion balance (see p. 18).

In principle, small pieces of well-washed blotting-paper (about 15 mm. × 25 mm. in size) are clamped in the pincer attachment and weighed on the torsion balance. The weight should be of the order of 120–150 mg. About 0·1 ml. of blood is then pipetted on the paper, allowed to spread, and reweighed. The difference between the two weights gives the exact quantity of blood taken. The paper containing the blood is then placed in a small stoppered tube and covered with an "extraction fluid" which fulfils the dual task of precipitating the proteins and extracting the substance to be estimated. The tube is placed aside for an appropriate period, the fluid poured off, and the estimation made.

It is convenient when using this system to carry out about ten to twelve estimations of the same constituent simultaneously, and by using a rack holding this number of tubes, each containing the appropriate quantity of extraction fluid, the time factor for the estimation may be reduced considerably.

Preparation of paper. Contamination due to impurities in the absorbent paper must be carefully avoided. A good brand of practically ash-free paper should be used, and the method of treatment depends upon the nature of the estimation.

Thus for *blood sugar* estimations the paper must be free from starch and other soluble reducing substances. The paper is cut in strips and washed first in acetic acid and then in distilled water for hour periods until the washings no longer give the starch-iodine reaction. The paper is finally air dried, cut in pieces, and kept in a stoppered glass bottle.

For *nitrogen* estimations the paper must be ammonia-free. It is therefore washed with distilled water until a test of the washings with Nessler's solution is negative.

For *fat* estimations the paper is extracted with boiling alcohol.

For *chloride* estimations the paper is washed with dilute acetic acid until a negative test with silver nitrate is given.

Example 1: Chloride.

PRINCIPLES. The paper on which the blood has been pipetted is maintained in 92% alcohol for 5 hours and the solution is then titrated with silver nitrate by Mohr's method, using potassium chromate indicator.

SOLUTIONS.

1. *92% alcohol (chloride-free)*.

2. *N/100 silver nitrate solution*.

3. *Potassium chromate, 7% solution*.

METHOD. About 100 mg. of blood are weighed on to a paper 25 mm.×15 mm. The paper is allowed to air dry for about 5 minutes and then is placed in a small Bang tube (a flat-bottomed test-tube 15 mm. diameter and 50 mm. high); 92% alcohol is poured into the tube until the level is about 5 mm. above the top of the paper. The cork is inserted and the tube placed aside for at least 5 hours (or conveniently until the following day). The alcohol is then carefully decanted into a titration flask (a conical pedestal glass is recommended) and the tube is washed out with 5 ml. of alcohol. To the combined alcohol solution is added 1 drop 7% potassium chromate solution and, after mixing, $N/100$ silver nitrate is run in from a Bang 2 ml. micro-burette until a change of colour from yellowish to a reddish-brown is just persistent. A blank titration is made on an equivalent amount of the alcohol; this value must be subtracted before computing the result.

Example 2: Nitrogen Fractions.

(a) Non-protein Nitrogen.

PRINCIPLES. The paper containing the blood is extracted with a phospho-tungstic acid mixture, which also precipitates proteins. The solution is then oxidised with sulphuric acid and the ammonia is distilled, using the Bang micro-Kjeldahl distillation apparatus, into a standard solution of acid iodate. Back-titration is made iodometrically.

SOLUTIONS.

1. *Phosphomolybdic reagent*. Dissolve 10 g. of sodium phosphomolybdate (ammonia-free) and 10 g. of sodium sulphate in 150 ml. water. Add 0·5 ml. of 30% caustic soda and heat for 15 minutes to boiling-point. Cool; transfer to a 2 l. measuring flask, add 30 g. of concentrated sulphuric acid and 0·5 of dextrose and make up to 2 l.

2. *Copper sulphate, 10% solution*.

3. *N/200 acid iodate solution*. Take 5 ml. of $N/10$ sulphuric acid and 20 ml. $N/10$ potassium iodate and dilute exactly to 100 ml. with water.

4. *Potassium iodide, 5% solution (freshly prepared)*.

5. *N/200 thiosulphate*. Dilute $N/10$ solution 5 ml. to 100 ml. prior to use.

6. *Starch solution.* Dissolve 1 g. of soluble starch by boiling in 20 ml. of water. Pour into 80 ml. of saturated potassium chloride solution in a cylinder, mix, and allow to stand over-night. Pour off as required.*

APPARATUS. Steam is generated from a 500 ml. flask *a* (fig. III.21). The micro-Kjeldahl flask *b* fixes on to a rubber bung, through which the tube from the steam generator passes almost to the bottom of the Kjeldahl flask. This connection from the steam generator is inclined downwards towards the flask and carries a thistle funnel with a double bulb trap through which is introduced caustic soda solution. The outlet from the Kjeldahl flask through the rubber bung is a glass steam trap *c*, and this passes into a
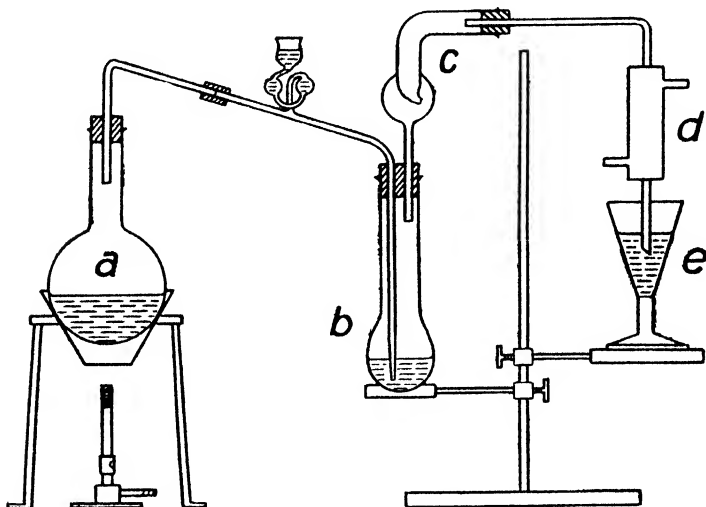
Fig. III.21. Bang's Apparatus for Kjeldahl Estimation of Nitrogen.

silver condenser *d*, the inner tube of which dips into a conical glass receiving flask *e*.

METHOD.

(i) *Protein removal.* About 100 mg. of blood are weighed on to the paper (25 mm. × 15 mm.) and, after 5 minutes' air drying, are transferred to the

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* A greatly improved indicator for iodometric analysis has been described by S. Peat, E. J. Bourne, and R. D. Thrower (*Nature*, 1947, **159**, 810). It consists of a solution of the sodium salt of starch glycollic acid and can be kept for months without deterioration. Since the blue iodine complex is water soluble, the indicator may be added at any stage of a titration.

To prepare the salt make a dispersion of 10 g. of starch in 160 ml. of water and 30 ml. of 50% NaOH, warm to 50° C. and slowly add a solution of 5 g. of sodium mono-chloroacetate in 20 ml. of water. Neutralise the product with acetic acid and dialyse in a cellophane bag against running water for 3 days. Then precipitate the white solid by adding an excess of alcohol, and purify it by extraction with 90% alcohol in a Soxhlet.

The product is used as a 2% solution in distilled water.

Bang tube (50 mm. × 25 mm.) and covered to a depth of about 3–4 mm. with the phosphomolybdic reagent. The tube is stoppered and stood aside for 24 hours. The solution is then filtered, through a small paper, directly into a 50 ml. Pyrex micro-Kjeldahl flask; the tube is rinsed with 5 ml. of the same solution and this is poured through the same paper into the flask. Filtration is essential, since minute particles of protein precipitate which might become detached from the paper could otherwise give grossly erroneous results.

1 ml. of sulphuric acid is added to the flask, which is then heated on a Bunsen stand (fig. II.11, p. 78) until oxidation of organic matter is complete. This takes about 10–15 minutes, and heating should be gentle at first until all water is removed and then more vigorous until a canary-yellow solution of phosphomolybdic acid is obtained. After cooling, 10 ml. of distilled water are added to the flask and it is placed in position on the Kjeldahl distillation apparatus.

(ii) *Distillation.* 20·0 ml. of $N/200$ acid iodate solution are placed in the conical receiving flask. About 5 ml. of 20% caustic soda are introduced into the Kjeldahl flask *via* the thistle funnel. The receiver is placed in position and the condenser water turned on. Steam is now passed into the flask and distillation of the ammonia is allowed to proceed. The bulk of the ammonia is removed in the first minute, but the process is continued for 5 minutes. Then the receiver is lowered, the end rinsed with distilled water, the receiver is removed, and steam is disconnected.

(iii) *Titration.* 2 ml. of 5% potassium iodide solution are added to the solution, which is then covered with a watch-glass and allowed to stand for 5 minutes to allow the reaction

$$5KI + KIO_3 + 3H_2SO_4 = 3I_2 + 3K_2SO_4 + 3H_2O$$

to proceed to completion.

The solution is then titrated with $N/200$ thiosulphate from a Bang micro-burette until a pale straw colour. 2 drops of starch solution are then added and titration is continued until the blue starch colour disappears and remains absent for 5 minutes.

The $N/200$ acid iodate solution should be restandardised each time it is used. The thiosulphate factor must also be determined before each series of analyses.

(b) Blood Urea.

PRINCIPLES. Urea is extracted with alcohol/ether; the solution is then oxidised and the ammonia is measured by the Bang micro-Kjeldahl procedure as described above.

SOLUTIONS.

1. 50 : 50 *mixture of absolute alcohol and purified ether.*

2. *As for non-protein nitrogen.*

METHOD. About 100 mg. of blood are weighed into the paper (25 mm.×15 mm.), allowed about 5 minutes to air dry, placed in the Bang tube, covered to a depth of about 3 mm. with alcohol/ether mixture, corked, and allowed to extract for 24 hours. Extraction is very slow and cannot be readily curtailed. The alcohol/ether solution is filtered into a Kjeldahl flask, the tube is washed with 3 ml. of alcohol/ether, and this also is poured through the filter into the flask. A few drops of 10% copper sulphate are added and the bulk of the alcohol/ether is removed by heating on a water-bath. 1 ml. of concentrated sulphuric and 5 ml. of water are added, and the rest of the alcohol is boiled off. Oxidation of organic matter, distillation of ammonia, and titration are then carried out as described on p. 199.

B. Bloor's System for Micro-determination of Fats*

Bloor's methods for the determination of the fat fractions in blood are micro-titration procedures after extraction and subsequent isolation of the required components. Advantage is taken of the fact that when plasma is pipetted into a mixture of alcohol and ether, precipitation of the proteins occurs and at the same time fatty substances dissolve. This extract is treated variously to separate the fat fractions, which are then oxidised with an excess of chromic acid. Back-titration is made iodometrically after the method of Bang.

Preparation of the Alcohol/Ether Extract

REAGENT.

3 parts of 95% alcohol to 1 part of redistilled ether.

METHOD. Introduce 40 ml. of the mixture into a 50 ml. graduated flask. Add slowly, with rotation, 2·50 ml. of plasma or serum; shake well. Immerse the flask in a boiling water-bath, and agitate it occasionally until the mixture begins to boil. Leave for a few seconds longer, then cool to room temperature. Make up to 50 ml. with alcohol/ether, thoroughly mix by shaking, and then filter through a fat-free filter-paper. Keep the filtrate stoppered to avoid evaporation.

20 ml. of filtrate=1 ml. of plasma.

Example 1: Estimation of Total Lipoid Fatty Acids Plus Cholesterol.†

SOLUTIONS.

1. $N/10$ thiosulphate solution.

2. N $(M/6)$ potassium dichromate solution.

3. Potassium iodide, 10% solution, freshly prepared

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* Bloor, W. R., *J. Biol. Chem.*, 1928, **77**, 53.
† Bloor, W. R., ibid.

4. *Silver chromate in concentrated sulphuric acid*. Dissolve 5 g. of silver nitrate in 25 ml. of water in a 100 ml. centrifuge tube, add 50 ml. of 10% potassium chromate solution, and centrifuge. Wash the precipitate well with water until free from nitrates and then dissolve in 500 ml. of concentrated sulphuric acid.

5. *Distilled petroleum ether: b.p.* 40°–60° C.

6. *Sodium ethylate*. Dissolve 2 g. of freshly cut sodium in 100 ml. of absolute alcohol, keeping the solution cool in the process. Keep at 0° C. in the dark and discard when much discoloured.

7. *Dilute sulphuric acid*. 1 volume H_2SO_4+3 volumes of water.

METHOD.

(i) *Saponification*. 20·0 ml. of alcohol/ether extract are pipetted into a 100 ml. Erlenmeyer flask and 2 ml. of sodium ethylate are added. The mixture is evaporated on a water-bath until the bulk is reduced to about 2 ml. and the residual alcohol is removed in a current of air. The pasty residue is treated with 1 ml. of dilute sulphuric acid to liberate the free fatty acid and heated on a water-bath for 1 minute; 10 ml. of petroleum ether are then added, and the mixture is allowed just to boil. The flask is rotated for about 3 minutes while the solvent is boiling, and the latter is then poured as completely as possible into a 25 ml. volumetric flask. The heating and extraction are repeated with further 5 ml. portions of petroleum ether, the sides of the flask are washed down, and the petrol is poured off completely into the volumetric flask. After cooling to room temperature the flask is filled to the mark, stoppered, and its contents are mixed.

(ii) *Oxidation*. A 10 ml. portion of the petroleum ether is pipetted into a 125 ml. stoppered flask. The solvent is evaporated, the last traces being removed in a current of carbon dioxide. 5·0 ml. of silver chromate reagent followed by exactly 3·0 ml. of N dichromate solution are added, the flask being rotated meanwhile. At the same time as the estimation is being made, a blank determination is run on the reagents by submitting them to exactly the same procedure as the blood filtrate. The flasks are then loosely stoppered and placed for 5 minutes in an electric oven regulated to 124° C. The flasks are then agitated to mix, tightly stoppered, and then returned to the oven for 10 minutes. The mixture should retain some brownish tinge throughout. If it becomes completely green, exhaustion of the reagent has occurred; then a further amount of silver chromate and dichromate should be added and the mixture heated again in the manner described above. Alternative to the oven procedure, a steam-bath may be used, in which case the heating is continued for 90 minutes. At the end of the heating period the contents of the flasks while still hot are diluted with 75 ml. of distilled water.

(iii) *Titration*. After cooling, 10 ml. of potassium iodide solution are added and $N/10$ thiosulphate is run in from a burette. The solution changes during the titration from a muddy greenish-brown to a light blue. At this stage a few drops of starch are added and the titration is continued until the starch blue colour disappears.

      1 mg. palmitic acid requires   3·57 ml. of $N/10$ dichromate.

      1 mg. oleic acid requires   3·61 ml. of $N/10$ dichromate.

      1 mg. cholesterol requires   3·92 ml. of $N/10$ dichromate.

Assuming that blood contains twice the quantity of fatty acids as cholesterol, 3·7 ml. is taken as the $N/10$ dichromate factor equivalent to 1 mg. of fatty acids plus cholesterol.

Example 2: Estimation of Cholesterol After Separation as Digitonide.

PRINCIPLES. The alcohol/ether extract of Bloor is treated with digitonin; the cholesterol digitonide is separated off and estimated by the dichromate oxidation technique.

SOLUTIONS.

1. *Sodium ethylate*, 20%. Freshly prepared from metallic sodium and absolute alcohol.

2. *Light petroleum*, *b.p.* 40°- 60° C.

3. *Digitonin solution*. Dissolve 1 g. of digitonin in 50 ml. of absolute alcohol. Add 50 ml. of water. Stand over-night and filter off any precipitate which forms.

4. *Acetone*.

5. *Aluminium chloride*, 4% *solution*.

6. *Ammonia*, 50% *by volume*.

7. *Hydrochloric acid*, 30% *by volume*.

METHODS.

(i) HYDROLYSIS AND SEPARATION OF THE DIGITONIDE.*

(*a*) *Free cholesterol*. 10 ml. of alcohol/ether extract (twice filtered through the same paper) are transferred to a 50 ml. wide-mouthed Erlenmeyer flask and 0·5 ml. of 1% digitonin solution is added. The solution is then evaporated to dryness over a water-bath, the last traces of water being removed in a current of air. 2 ml. of water are then added to the flask and the contents are brought to boiling. The colloidal solution which forms is broken up by the addition of 4 ml. of acetone, which dissolves the fat. The contents of the flask are then transferred to a centrifuge tube— a further 3 ml. of acetone are added to the flask, the sides of which are rubbed with a rubber policeman, and the acetone is then added to that already in

* Obermer, E., and Milton, R. F., *Biochem. J.*, 1933, **27**, 345.

the centrifuge tube.   One drop of aluminium chloride is added and, after mixing, 1 drop of ammonia solution to precipitate the aluminium as hydroxide.   The tube is then centrifuged for 1 minute at high speed.   As the aluminium hydroxide is thrown down, so the digitonide is taken with it.   The supernatant liquid is decanted off completely and the hydroxide is dissolved in 1 drop of 30% hydrochloric acid.   The original precipitation flask is rinsed with a further 3 ml. of acetone, which is then transferred to the centrifuge tube.   After mixing, the tube is again centrifuged, this time for 5 minutes at about 3,000 r.p.m.   The supernatant liquid is decanted off and the precipitate is washed again with 3 ml. of acetone and finally with 3 ml. of ether.

The ether is removed by decantation, and the precipitate is broken up and warmed to remove residual ether completely.

The pure cholesterol precipitate is now ready for estimation by the oxidation procedure.

(b) *Total cholesterol.* 5·0 ml. of alcohol/ether extract, to which is added 0·2 ml. of sodium ethylate, are heated under reflux in glass-joint micro-apparatus for 30 minutes.

At the end of this time the bulk of the alcohol is removed by distillation until about 2 ml. of fluid remain in the flask.   15 ml. of petroleum ether are then added and, after bringing to the boil, 2 ml. of water are added and the contents of the flask are well shaken.   The petroleum layer is then poured through a filter-paper into a dry Erlenmeyer flask.   The residual water layer is shaken with a further 10 ml. of petroleum ether, which is passed through the filter-paper as before.   This process is repeated; then to the combined extracts is added 0·5 ml. of digitonin solution, and the bulk of the petroleum is distilled off.   10 ml. of alcohol/ether are then added, and the contents of the flask are evaporated to dryness on a water-bath, the last traces of solvent being removed in an air current.   The separation of the cholesterol as digitonide is then carried out exactly as for free cholesterol.

Precautions.

(a) The alcohol/ether extract should be neutral.   Acid inhibits digitonide formation.

(b) The hydrolysate should not be allowed to dry in the presence of alkali at any stage.   Then the cholesterol is so altered that digitonide formation does not occur.

(ii) Oxidation of the Digitonide.*

To the cholesterol digitonide precipitate is added silver chromate and dichromate in the manner described on p. 201.   The precipitate is broken up with a small stirring rod and the tube is stoppered and heated, as described before, at 124° C. for 15 minutes or at 90° C. for 90 minutes.   After dilution with 75 ml. of water and cooling, potassium iodide solution is added and the liberated iodine is titrated with thiosulphate.

* Okey, R., *Proc. Soc. Exp. Biol.*, 1929, **26**, 518.

Example 3: Estimation of Phospholipoids.*

PRINCIPLES. Phospholipoids are precipitated from the alcohol/ether extract by acetone and magnesium chloride, and the lipoid in the precipitate is oxidised by the dichromate procedure.

SOLUTIONS.

The reagents required for estimation of total lipoids (see p. 200) together with:

1. *Redistilled acetone.*

2. *Saturated solution of magnesium chloride in absolute alcohol.*

3. *Moist peroxide-free ether.* Test for peroxides by adding potassium iodide solution. No yellow colour should appear. Store in a dark bottle in a cool cupboard.

METHOD.

(i) *Isolation of Phospholipoids.* 20 ml. of alcohol/ether extract from plasma are evaporated to dryness in a small beaker. The residue is extracted three times, with about 3 ml. of petroleum ether each time, and the extracts are united in a 15 ml. centrifuge tube. The tube is centrifuged, and the clear liquid is transferred to another tube, placed in a water-bath, and the petroleum extract is evaporated down to about 2 ml. Bumping is avoided by using a capillary tube inserted into the liquid. To the cooled solution are now added 7 ml. of acetone and 0·1 ml. of magnesium chloride solution. The contents of the tube are thoroughly mixed and the phospholipoid precipitate is centrifuged down. The acetone is decanted off and the precipitate is washed with a further 7 ml. of acetone and, after centrifuging, is allowed to drain dry by inverting over a filter-paper.

The precipitate is then dissolved in 5 ml. of pure ether, solution being aided by breaking the mass with a glass rod. The tube is centrifuged and the undissolved residue together with the globule of magnesium chloride solution are thereby separated from the ether solution. The ether solution is quantitatively transferred to an oxidation flask (p. 201), the ether washings from the tube being added also. The solvent is now removed by evaporation and the last traces are thoroughly blown off in a current of air.

(ii) *Oxidation.* Silver chromate solution and standard dichromate are then added exactly as described on p. 201. The flask is then heated at 124° C. for 15 minutes or at 90° C. for 90 minutes. At the end of this time 75 ml. of water are added. After cooling, potassium iodide solution is added and the liberated iodine is then titrated with thiosulphate. A blank experiment is carried out at the same time, using 20 ml. of pure alcohol/ether mixture in place of the blood extract. The figure so obtained must be subtracted from that given by the plasma extract.

1 mg. of phospholipoid ≡ 3·0 ml. of $N/10$ dichromate.

* Bloor, W. R., *J. Biol. Chem.*, 1929, **82**, 273.

C. MICRO-DIFFUSION METHODS OF ANALYSIS*

The application of the principles of micro-diffusion to analytical technique has been studied very extensively by Conway. He has devised a special micro absorption apparatus and by using it has elaborated techniques covering a number of the most common analytical requirements. The procedures are very neat, simple to carry out, require a minimum of apparatus, laboratory space, and manipulative time, are particularly suitable for mass analysis, and are capable of a very high degree of accuracy.

CONWAY'S STANDARD MICRO-DIFFUSION CELL (fig. III.22) consists of a petri dish 6 cm. in diameter and 1 cm. deep, in the centre of which is fused a piece of glass tubing, 3·5 cm. diameter and 0·5 cm. deep, to form an inner cell or compartment. The edge of the outer dish is ground true so that a lid in the form of a square of flat ground-glass may enclose completely any gases in the dish. The lid is suitably smeared with vaseline-paraffin mixture to assist the seal.

In principle, the substance to be analysed is placed in the outer compartment and is acted upon by some reagent to produce a gas (e.g. ammonia

Fig. III.22. Conway's Micro-diffusion Cell.

or carbon dioxide), which diffuses into a known amount of standard acid or alkali previously placed in the inner compartment. Back-titration is then carried out to ascertain the amount of diffused gases.

Conway investigated all the factors affecting this type of gaseous diffusion and stated those giving optimum and reproducible results. With the standard type of apparatus the rate of absorption has been found to vary with (a) time, (b) fluid volumes, (c) salt content in outer chamber, (d) temperature, (e) agitation, and (f) depth of solution in the outer chamber.

Since, theoretically, diffusion is never complete, it becomes essential to standardise rigidly the conditions of the method in order to obtain reliable results, and although these may in the main be predicted by mathematical treatment, it is far better to subject standard solutions of the substances to be estimated to the proposed technique in order to ascertain the conditions for optimum diffusion.

Methods have been worked out for the micro estimations of ammonia, total nitrogen, urea, adenyl-phosphoric acid, amines, halogens, carbonates, and also for qualitative tests for alcohol and acetone.

* Conway, E. J., "Micro-diffusion Methods and Volumetric Error" (Crosby Lockwood, London, 1939; revised edition, 1947).

On account of the small quantities involved, extreme care must be taken in cleaning the glassware used, and especial attention paid to the selection of volumetric apparatus. For this purpose Conway devised the special burette described on p. 149. The errors concerned in micro-volumetric titration (see p. 136) should be carefully considered by users of these techniques, which may have wider applications than the particular analyses which are exemplified below.

Example 1: Estimation of Ammonia in Blood.*

PRINCIPLES. Human blood contains very little ammonia in a preformed condition, although this amount appears to increase after the blood is shed, due mainly to breakdown of adenyl-phosphoric acid. After about 5 minutes *in vitro* 50 μg. of ammonia nitrogen per 100 ml. is found. By the Conway method this quantity (i.e. 0·25 μg. of N) may be accurately measured in only 0·5 ml. of blood. The blood is treated with potassium carbonate and the liberated ammonia is allowed to diffuse into standard acid which is then back-titrated with standard baryta solution.

SOLUTIONS.

1. *Ammonia-free potassium oxalate.* Evaporate a solution of potassium oxalate, made alkaline to pH 11 with soda, until crystals form. Filter off the crystals on a Buchner funnel and keep in a stoppered bottle after desiccation.

2. *Fixative.* Dissolve 2 parts of paraffin wax (m.p. 40° C.) in 1 part of liquid paraffin.

3. *Standard solution of ammonia.* 0·471 g. of pure $(NH_4)_2SO_4$ in 1 l. of distilled water.

4. $N/5,000$ *hydrochloric acid.*

5. $N/2,500$ *barium hydroxide.*

6. *Indicator.* 4 volumes of 0·1% alcoholic solution of methyl red, together with 1 volume of 0·1% aqueous methylene blue. 1 ml. of this indicator is contained in every 100 ml. of standard acid.

7. *Potassium carbonate* (*saturated solution*). Boil to remove ammonia.

Preparation of the apparatus. The micro-diffusion apparatus must be scrupulously clean. To ensure this the following technique is used: (i) Wash *well* first with hot and then with cold tap-water; (ii) remove any grease by scrubbing with a test-tube brush, using a little soapy water, and thoroughly rinse; (iii) fill with $N/200$ (approximately) sulphuric acid containing a little indicator and stand for 15 minutes; (iv) well rinse with cold tap-water and finally with distilled water. Dry by shaking and draining—never use a cloth for this purpose.

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* Conway, E. J., *Biochem. J.*, 1935, **29**, 2755; "Micro-diffusion Analysis," 1947 edn., p. 99.

METHOD. 0·70 ml. of $N/5,000$ acid is pipetted into the central chamber of the apparatus; 0·5 ml. of saturated carbonate is placed in the outer chamber. The cover-plate is smeared with fixative, then placed in position and allowed to stand for 20 minutes before use to ensure diffusion of any ammonia impurity from the carbonate or fixative. Three similar units are prepared at the same time (a) for control, (b) for standard solution of ammonia, (c) for the blood.

After 20 minutes 0·50 ml. of standard solution is introduced into the outer chamber of unit (b) and 0·50 ml. of freshly shed oxalated blood into unit (c). The lid is made fast and the apparatus is rotated about fifteen times to ensure mixing in the outer compartments without spilling into the centre one, and then is set aside at room temperature for 10 minutes (accurately timed). The lid is removed and the excess of acid in the inner chamber is titrated with $N/2,500$ barium hydroxide solution from the Conway burette protected by a guard tube. The end-point of the titration is a neutral grey tinge.

The control unit and the one containing the standard solution of ammonia are titrated at the same time. Diffusion of ammonia under these conditions is not complete in this time period. In fact, only about 83% of the ammonia passes into the acid. Since, however, during 10 minutes the diffusion of the ammonia is linear, a proportional comparison with the amount given by the standard solution will give a very accurate result. This is more accurate than that obtainable by allowing the diffusion time to be increased, since there is a steady formation of ammonia in blood in the presence of alkali.

Example 2: Determination of Total Carbon Dioxide in Blood.

PRINCIPLES. Carbon dioxide is liberated from the outer compartment with acid and allowed to diffuse into a standard amount of barium hydrate contained in the inner department. The excess is titrated with standard acid to thymol-phthalein indicator.

SOLUTIONS.

1. 0·179N *hydrochloric acid.* Dilute 35·9 ml. of $N/1$ HCl to 200 ml. with distilled water. Each 0·01 ml. will be equivalent to 0·02 c.c. of carbon dioxide at N.T.P.

2. *Barium hydroxide solution.* Mix 44·9 ml. of $N/5$ barium hydroxide with 5 ml. of 0·1% thymol-phthalein and dilute to 100 ml.

3. $N/20$ *sodium carbonate solution.*

METHOD. Blood must be collected anaerobically for this estimation. This is achieved by allowing blood direct from the vein to flow under a layer of paraffin oil contained in a waxed centrifuge tube. A small amount of heparin is placed in the tube to prevent coagulation. Some low-melting-point wax is poured on the top to seal and the tube is centrifuged.

The diffusion unit is prepared as follows: 0·2 ml. of about N sulphuric acid is run into the outer chamber of the Conway unit. The lid is lightly coated with vaseline and placed in position. It is just slipped aside and 1 ml. of barium hydroxide solution is delivered into the central chamber from a micro-burette fitted with a guard tube. The apparatus is then tilted by placing a match-stick under one side, and at the appropriate moment the lid is pushed aside and 1 ml. of the separated blood plasma is introduced into the outer chamber *via a syringe pipette*—a necessary precaution to avoid loss of carbon dioxide which would occur with sucking type of burettes. The apparatus is then rotated to mix the outer chamber contents, the lid is pressed well into position, and the unit set aside for 60 minutes to allow diffusion to go almost to completion.

At the end of this period the contents of the inner chamber are titrated with standard hydrochloric acid from a micro-burette until the thymol blue indicator is just colourless (i.e. about pH 9·3), at which point the excess of barium hydroxide is neutralised. The titration should be performed as quickly as possible so as to avoid (*a*) absorption of carbon dioxide from the air and (*b*) action of acid upon the barium carbonate. Comparison should be made with standard carbonate solution treated in the same manner.

The method is suitable for quantities of carbon dioxide from 0·2–1 mg.

Example 3: Micro-estimation of Bromide in the presence of Chloride and Iodine.

PRINCIPLES. The material to be analysed is ashed. The bromide is oxidised to bromine, which diffuses into a potassium iodide solution and the liberated iodine is titrated with thiosulphate.

REAGENTS.

1. *Pure powdered potassium dichromate.*
2. *N sulphuric acid.*
3. *Sulphuric acid, 40% by volume.*
4. *Potassium iodide, 20% solution, freshly prepared.*
5. *$N/200$ sodium thiosulphate.*

METHOD. 12 ml. of methyl alcohol are pipetted into a centrifuge tube and 1·00 ml. of blood is added, drop by drop, and well mixed. After centrifuging, 10 ml. of the clear fluid are transferred to a small crucible together with a quantity of a standard solution of potassium bromide containing exactly 2·00 mg. of bromide. (The bromide is added to ensure that the amount analysed lies on a linear diffusion curve.) The crucible is dried at 100° C. and heated over a Bunsen to a dull red for 1 minute. After cooling, its sides are washed down with 0·5 ml. of distilled water and the solution is pipetted into the outer chamber of the unit. The crucible is

rinsed with a further 0·5 ml. of water and this is added also to the outer chamber. A few carbon particles do not interfere. 0·2 g. of potassium dichromate is then added to the outer chamber followed by 0·1 ml. of *N* sulphuric acid.

The unit is then stood open on a bench for $1\frac{1}{2}$ hours and in this way any iodide which might be present is liberated and diffuses away.

1 ml. of 20% potassium iodide is now placed in the inner chamber and the greased lid is placed in position. The unit is then tilted, the lid is slightly displaced, and 1 ml. of 40% (by volume) sulphuric acid is quickly run into the outer chamber. The lid is quickly replaced, the fluids in the outer chamber are mixed by rotation, and the apparatus is stood aside for 2 hours. At the end of this time the liberated iodine is titrated to a starch end-point with *N*/200 thiosulphate from a micro-burette. Allowance should be made for the amount of bromine added and the method should be checked against standard solutions of bromide. If the amount of bromide present is less than 80 µg. it is recommended that the liberated iodine be measured colorimetrically, using micro-cups.

With these concentrations of acid and dichromate, chlorine is not appreciably liberated concomitantly with bromine. In fact, after 24 hours' action the amount of chlorine does not exceed 1 µg. if the solution is not greater than 1% with respect to sodium chloride.

The method can, however, be used for chlorine. For this, in place of dichromate and sulphuric acid as an oxidiser, half-saturated potassium permanganate in 30% sulphuric acid should be used.

D. Titrimetric Estimation of Blood Sugar*

The ferricyanide method of blood sugar estimation is the most reliable method for evaluating true glucose, rather than total reducing substances, in blood.

With appropriate modification it can be applied to extracts from foods, plants, etc.

PRINCIPLES. Potassium ferricyanide is quantitatively reduced by glucose in alkaline solution. The excess ferric salt is determined iodometrically after precipitation of the ferrocyanide as the zinc salt.

SOLUTIONS.

1. *N*/10 *sodium hydroxide*.

2. *Zinc sulphate*, 0·45% *solution*.

3. *N*/200 *alkaline ferricyanide*. Dissolve 1·65 g. of purified potassium ferricyanide and 10·6 g. of anhydrous sodium carbonate in 1 l. of water.

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* Hagedorn, H. C., and Jenson, B. N., *Biochem. Zeit.*, 1923, **135**, 4. For a colorimetric application of this reaction, see p. 334.

4. *Sodium chloride/zinc sulphate solution.* Dissolve 50 g. of sodium chloride in 150 ml. of water.   Dissolve 10 g. of zinc sulphate in 50 ml. of water: mix.

5. *Potassium iodide, 5% solution.*

6. *Acetic acid, 3% solution.*

7. *N/200 thiosulphate.* Dilute 5 ml. of N/10 thiosulphate to 100 ml. with water.

8. *Starch solution.* See p. 198.

METHOD. Into a test-tube containing 5 ml. of 0·45% zinc sulphate and 1 ml. of N/10 sodium hydroxide, 0·10 ml. of blood is pipetted by means of an Ostwald pipette, which is washed out with the solution.   After mixing,* the tube is placed in a boiling water-bath for exactly 4 minutes, cooled, and then the liquid is filtered, through filter-paper which has been washed free from all reducing substances, into a tube 30 mm. by 90 mm.   The precipitate on the paper is washed three times with 3 ml. of distilled water.

To the combined filtrates are added exactly 2·00 ml. of the ferricyanide solution (e.g. from a Krogh pipette, see p. 145).   The tube is then immersed in boiling water for exactly 15 minutes and then immediately cooled to room temperature.

At the same time as a determination is made, a blank is prepared using identical quantities of reagents, but omitting the addition of blood.   The two tubes are heated side by side and are cooled in the same manner.   To each solution are then added 3 ml. of zinc sulphate/sodium chloride solution, 1 ml. of 5% potassium iodide solution (freshly prepared), and then 2 ml. of acetic acid solution.   The solutions are then titrated with N/200 thiosulphate from a Bang micro-burette until the yellow colour is just perceptible.   2 drops of starch are then added and the titration is continued until the blue colour finally disappears.

CALCULATION. The titration figures of blank and unknown are corrected to N/200 (after titrating the thiosulphate against an N/200 acid-iodate standard) and then subtracted.

If the difference be A, then the glucose content per 100 ml. is given by the equation

$$Mg.\ per\ 100\ ml.\ blood = 1000 \left(0 \cdot 1735A + \frac{0 \cdot 005A}{2 \cdot 27 - A} \right)$$

E. ESTIMATION OF LACTIC ACID IN BLOOD†

This reaction involves controlled oxidation of lactic acid to acetaldehyde, and illustrates how, on the micro-chemical scale, one can separate one component from a very complex system.

* Do not shake a tube with the thumb over the top.   Quite appreciable amounts of reducing substances may be introduced in this manner.
† Friedmann, T. E., Cotonio, M., and Shaffer, P. E., *J. Biol. Chem.*, 1927, **73**, 335.

PRINCIPLES. Blood is deproteinised with tungstic acid; glucose and other substances are removed from the filtrate with copper and lime. The lactic acid in solution is then oxidised with a permanganate mixture to acetaldehyde, which is distilled into bisulphite, and excess bisulphite is destroyed by titration with iodine. The aldehyde-bisulphite compound is then split up by addition of bicarbonate and the bisulphite set free is accurately titrated with standard iodine.

APPARATUS. The apparatus (fig. III.23) consists of boiling-flask a, condenser b, and absorber c, made from Pyrex glass. The flask is of 250 ml. capacity and is fitted with a rubber bung into which passes a condenser and a glass tube to which a funnel d is attached by means of a piece of rubber tubing.
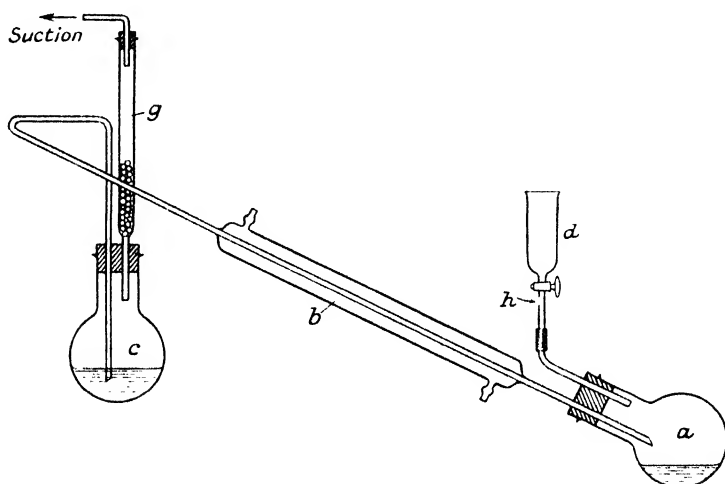
Fig. III.23. Distillation of Acetaldehyde from Lactic Acid.

Below the tap of the funnel is a small hole h through which air may enter. The condenser (about 40 cm. long) leads into an absorption flask which is guarded by an absorption tower g. Suction is applied from the top of the absorber.

SOLUTIONS.

1. *Sodium tungstate*, 10% solution.

2. *N/12 sulphuric acid.*

3. *Copper sulphate*, 25% solution.

4. *Powdered calcium hydroxide.*

5. *Phosphoric acid solution.* Dilute 135 ml. of syrupy phosphoric acid to 1 l. with water.

6. *Manganous sulphate*, 10% solution.

7. *Oxidising solution.* Add 0·1% manganous sulphate solution to $N/100$ potassium permanganate solution until the purple colour is almost discharged.

8. *Sodium bisulphite,* 1%.

9. *$N/500$ Iodine.* Dilute 20 ml. of $N/10$ iodine solution to 1 l.

10. *Sodium bicarbonate solution.*

11. *Starch solution* (p. 198).

METHOD.

(a) *Lactic acid separation.* Blood freshly drawn, or that preserved by the addition of ammonium fluoride, is deproteined with tungstic acid. To 2·0 ml. of blood are added slowly 16 ml. of $N/12$ sulphuric acid and then slowly with stirring 2 ml. of 10% neutral sodium tungstate solution. After mixing and standing for 15 minutes the mixture is filtered through dry paper and a clear filtrate should result.

10·0 ml. of the filtrate are treated with 1·0 ml. of 25% copper sulphate solution and enough dry calcium hydroxide to make the solution alkaline. The mixture is shaken at intervals during 30 minutes and then is filtered twice through the same filter-paper, which has previously been well washed. 5·0 ml. of the filtrate (i.e. 5/11ths of the original filtrate) are then transferred to the boiling-flask.

(b) *Oxidation.* To the boiling-flask are now added 5 ml. of $2M$ phosphoric acid, 10 ml. of 10% manganous sulphate, and distilled water up to about 100 ml. A pinch of talc is added as an anti-bump. The flask is connected to the condenser, which is in turn connected to the receiving flask without the bead tower. The liquid is then heated for 5 minutes (with air drawn through) to remove volatile substances such as acetone which might affect the titration. A fresh receiving flask and the bead tower (see fig. III.23) are then put in place. About 10 ml. of 1% bisulphite solution are then placed in the receiving flask. The apparatus is again attached to suction and air is drawn through at a rate of about 2 l. per minute.

Heating is carried out with a micro-burner and $N/100$ manganese dioxide suspension (reagent 7 above) is dropped into the boiling-flask at the rate of about 4 ml. per minute until an excess (as denoted by the pink colour) is present in the flask. The pink colour should persist for about 10 minutes before it is considered than an excess is present. Heating and aeration is continued in all for 20 minutes, timed from the commencement of the addition of the manganese dioxide. The process is then stopped, the receiver lowered, and the tower rinsed six times with 5 ml. of water.

(c) *Claisen titration.* The free bisulphite is now titrated with $N/10$ iodine until on the addition of a few drops of starch a definite blue persists. The excess iodine is then removed by the addition of 1 drop of $N/10$ thiosulphate. $N/500$ iodine is now added, drop by drop, until a faint blue

colour is just discernible. The bisulphite which is combined with the aldehyde is now set free by addition of about 1 g. of sodium bicarbonate. $N/500$ iodine is then added from a 2 ml. Bang micro-burette until a blue colour persists for at least 1 minute.

1 ml. of $N/500$ iodine $\equiv \cdot 09$ mg. of lactic acid.

F. MICRO-ESTIMATION OF IODINE IN ORGANIC MATTER*

This method is suitable for the estimation of traces of iodine, 0·5 μg. being measurable to 10% accuracy.

PRINCIPLES. The organic matter is destroyed with chromic/sulphuric acid. At the same time the iodine is changed quantitatively to the non-volatile iodic acid. The iodic acid is then reduced to elementary iodine by the action of phosphorous acid. The iodine is distilled into alkali and titrated in the usual manner with thiosulphate.

The alcohol-soluble iodine compounds (which include the inorganic compounds) and the alcohol-insoluble compounds (which include such organic compounds as active thyroid substances) may be separated and the different fractions determined.

REAGENTS.

1. *Iodine-free water.* Redistil water from A.R. potassium hydroxide solution. Store in stoppered Pyrex bottles.

2. *Sulphuric acid, 3%.* Boil 30 ml. concentrated sulphuric acid for 30 minutes with 2 drops 30% hydrogen peroxide. Dilute to 1 l. with iodine-free water.

3. *Ceric sulphate, 5%.* Dissolve 10 g. anhydrous ceric sulphate and dilute to 200 ml. with 3% sulphuric acid.

4. *Chromic acid solution.* Dissolve 250 g. of A.R. chromic acid in 150 ml. of iodine-free water.

5. *Phosphorous acid, 50%.* Dissolve 250 g. of anhydrous reagent phosphorous acid in iodine-free water and dilute to 500 ml.

6. *Potassium hydroxide, 1M.* Dissolve 28·05 g. of potassium hydroxide and dilute to 500 ml. with iodine-free water.

7. *Bromine A.R. iodine-free.*

8. *Potassium iodide, 1%.* Dissolve 1 g. of iodate-free potassium iodide and dilute to 100 ml. with iodine-free water. Keep in the dark, in a refrigerator. Renew after 2 weeks.

9. *Starch solution* (see p. 198).

* Stevens, C., *J. Lab. and Clin. Med.*, 1936-37, **22**, 1074. Liepert, T., *Biochem. Zeit.*, 1933, **261**, 436; 1934, **270**, 448. Trevorrow, V., and Vashena, G., *J. Biol. Chem.*, 1935, **110**, 29; 1936, **114**, 351.

10. *N/2,000 sodium thiosulphate.* Prepare $N/10$ potassium bi-iodate and $N/10$ sodium thiosulphate. Standardise the thiosulphate against the bi-iodate each day and dilute 2·5 ml. to 500 ml. with iodine-free water. Keep all solutions in a refrigerator in the dark.

11. *Carborundum.* Boil small pieces (No. 6 mesh) for 10 minutes in dilute HNO_3; and then for 10 minutes with dilute phosphorous acid. Rinse and boil several times with iodine-free distilled water. Heat to redness for 5 minutes.

12. *Potassium oxalate.* Recrystallise by a single precipitation with ethyl alcohol from a saturated aqueous solution.

13. *Ethyl alcohol.* 95% iodine-free. Redistil alcohol from pure caustic potash solution.

APPARATUS. The apparatus (fig. III.24) which has interchangeable ground-glass joints, consists of a 500 ml. Pyrex digestion flask, a 10 ml. funnel, and a connecting arm with trap and a condenser. The receiver is a 100 ml. Pyrex extraction flask.

METHOD.

(*a*) *Fractionation.* 10 ml. of oxalated blood are added to 100 ml. of ethyl alcohol, mixed thoroughly, and filtered through a fine paper. The residue is washed three times with 15 ml. alcohol. To estimate "organic iodine" the residue on the filter-paper is

Fig. III.24. Micro-distillation of Iodine.

treated as for whole blood, whilst to estimate "ionised iodine" the filtrate is evaporated to dryness before proceeding.

(*b*) *Oxidation.* 10 ml. of blood are transferred to the digestion flask, the neck of which is rinsed down with 10 ml. of iodine-free water, and a piece of carborundum is added. 17 ml. of chromic acid solution are pipetted into the flask quickly followed by 100 ml. of concentrated H_2SO_4; adding this at first in portions of a few millilitres and then more rapidly after much chromic acid precipitates. The flask is shaken between additions, cooling all the

time under a tap. 1 ml. of ceric sulphate solution is then added and the flask is at once heated moderately. The addition of reagents should take not more than 3 minutes to avoid losses of iodine.

Heating is continued until bubbling due to decomposition of chromic acid has almost ceased and white fumes are evolved when a few millilitres of water are added. This should take approximately 10 minutes. If the digest becomes too hot (above 210° C.) bumping and precipitation of chromic sulphate occur.

When digestion is complete, 100 ml. of iodine-free distilled water are added cautiously by pouring directly on to the digest to ensure good mixing. As soon as bubbling has ceased the funnel is inserted and the flask fitted to the condenser. 1·00 ml. of molar potassium hydroxide solution is added to the receiving flask and the condenser tip is immersed. 6 ml. of 50% phosphorous acid solution are transferred to the funnel and added cautiously to the contents of the digestion flask. After rinsing down the funnel with 30 ml. of iodine-free water, the stopcock is closed and the contents of the flask are brought gently to the boil and then heated more strongly. The digested mixture should not be allowed to cool before this distillation is begun. Distillation is continued until white fumes appear at the top of the condenser, by which time (ca. 30 minutes) about 100 ml. of distillate will have come over. During the distillation the contents of the receiver are constantly evaporated so that the volume of distillate has, meanwhile, been kept at about 5 ml. The receiving flask is then removed, after washing-in from the condenser-tip with a few millilitres of iodine-free water.

(c) *Titration.* The distillation is made up to 15 or 20 ml. by rinsing down the walls of the receiver. 1 drop of 0·02% methyl orange and then 3% sulphuric acid are added, drop by drop until the colour is a clear pink, with no trace of orange. Bromine vapour is poured in from the mouth of a bottle containing bromine until the solution assumes a light yellow colour on stirring. Excess of bromine is boiled off and the solution is evaporated to 2 ml. After cooling to room temperature 4 drops of 1% potassium iodide and 1 drop of 1% starch are added. The free iodine is then titrated with $N/2,000$ sodium thiosulphate, delivered in 0·005 ml. portions from a syringe pipette (p. 152) the tip of which is immersed in the solution being titrated.

A blank is run on the reagents to correspond with amounts used in the analysis. This blank should not indicate more than 0·25 μg. of iodine.

SUMMARY OF METHODS SUITABLE FOR MICRO-VOLUMETRIC ESTIMATION OF INORGANIC SUBSTANCES

Substance	Method	Reference
Aluminium.	Separation with 8-hydroxyquinoline and titration with standard bromate and thiosulphate.	Monier-Williams, G. W., "Aluminium in Food," Ministry of Health Report, 1935. [H.M.S.O.]
Antimony.	Distillation as $SbCl_3$ and titration with bromate or distillation of $SbCl_3$ into $NaHCO_3$ and titration with standard iodine.	Rowell, J. Soc. Chem. Ind., 1907, **25**, 1181.
Arsenic.	Distillation as $AsCl_3$, evolution of AsH_3, absorption in $HgCl_2$ and titration with standard iodine in buffered solution.	Cassel, C., and Wichman, H., J. Ass. Off. Agr. Chem., 1939, **22**, 436.
Barium.	Titration with standard Na_2SO_4, using tetrahydroxyquinone as indicator.	Schroeder, Ind. Eng. Chem. (Anal. Edn.), 1933, **5**, 403 (see p. 186).
Boron.	Distillation in the presence of methyl alcohol as $(CH_3O)_3B$. After addition of mannitol titration is carried out with standard alkali to phenolphthalein end-point.	Richmond and Harrison, Analyst, **27**, 197; Wilcox, Ind. Eng. Chem., 1930, **2**, 358.
Bromine.	Oxidation with $HClO$ to $HBrO_3$. Excess $HClO$ removed with H_2O_2. Excess H_2O_2 is removed by boiling. Addition of KI liberates iodine thus: $HBrO_3 + 6HI \rightarrow 3I_2 + HBr + 3H_2O$. Titration is made with thiosulphate.	Dixon, Biochem. J., 1934, **28**, 48.
Cadmium.	Precipitation with sodium anthranilate and titration with bromide/bromate.	Shennan, Smith, and Ward, Analyst, 1936, **61**, 395.
Calcium.	Precipitation with oxalate and titration with permanganate.	See p. 167.
Cerium.	Oxidise to Ce^{IV} with sodium bis-muthate; filter. Add excess standard $FeSO_4$ and titrate excess with standard permanganate.	Metzer, J. Amer. Chem. Soc., 1909, **31**, 523.
Copper.	Separation with salicyl-aldoxime, hydrolysis to hydroxylamine and estimation with ferric salt and permanganate.	Hopkin and Williams, "Organic Reagents for Metals" (London, 1938), p. 114.
Chloride.	Titration with standard silver (Volhard). Titration with standard mercuric nitrate. Titration with thiosulphate.	p. 180. p. 182. p. 183.
Chromium.	(i) Oxidise to dichromate with bromine; precipitate with barium. Add KI and titrate with thiosulphate.	Scott and Furman, "Tech. Methods of Analysis," p. 288.

SUMMARY OF METHODS SUITABLE FOR MICRO-VOLUMETRIC ESTIMATION OF INORGANIC SUBSTANCES—Continued

Substance	Method	Reference
Chromium.	(ii) Oxidise with sodium peroxide. Acidify and boil to remove excess peroxide. Add excess ferrous sulphate and then titrate excess with standard dichromate, using diphenylamine indicator.	Scott and Furman, ibid., p. 291.
Cobalt.	Precipitation with KNO_2 as cobaltinitrite, $K_3Co(NO_2)_6$, and permanganate titration.	Christo Nikolow, *Przemysl Chem.*, 1933, **17**, 46.
Fluoride.	Titration with thorium nitrate using an alizarin indicator.	Willard and Winter, *Ind. Eng. Chem.* (Anal. Edn.), 1933, **5**, 7.
Gold.	Titration with hydroquinone in the presence of o-dianisidine.	Pollard, W., *Analyst*, 1937, **62**, 597 (see p. 178).
Iodine.	Oxidation with bromine to iodate. Addition of KI and titration with thiosulphate.	See pp. 89-90 and 215.
Iron.	Titration of ferrous salt with standard dichromate to diphenylamine indicator.	Knop, *J. Amer. Chem. Soc.*, 1924, **46**, 267 (see p. 172).
Lead.	Separation as chromate and titration with thiosulphate after addition of KI.	Fairhall and Keenan, *J. Amer. Chem. Soc.*, 1941, **63**, 3076.
Lithium.	Precipitation with potassium ferric iodate as $LiKFeIO_6$ and volumetric estimation of the iron.	Procke and Slouf, *Coll. Czech. Chem. Commun.*, 1939, **11**, 273.
Magnesium.	Separation as 8-hydroxyquinoline and titration with standard bromide/bromate.	See reference on Aluminium.
Manganese.	Oxidation with sodium bismuthate and titration with standard arsenite.	Scott and Furman, p. 563.
Mercury.	Addition of diphenylcarbazide and titration with standard NaCl until colour disappears.	See p. 182.
Molybdenum.	Titration with standard lead nitrate, using a diphenylcarbazone indicator.	Evans, *Analyst*, 1939, **64**, 1.
Nickel.	Separation as dimethylglyoxime. Dissolve in aqua regia. Titrate with cyanide.	Scott and Furman, p. 625.
Nitrates.	Reduction with Devarda's alloy. Distillation of ammonia into standard acid and titration of excess with soda.	Kolthoff, I., and Nopenen, G., *J. Amer. Chem. Soc.*, 1933, **55**, 1448.
Phosphorus.	Oxidation with nitric acid; precipitation as ammonium phosphomolybdate; titration with alkali.	See p. 164.
Potassium.	Precipitation as cobaltinitrite and permanganate titration.	See p. 168.

SUMMARY OF METHODS SUITABLE FOR MICRO-VOLUMETRIC ESTIMATION OF INORGANIC SUBSTANCES—Continued

Substance	Method	Reference
Selenium and Tellurium.	Selenates and tellurates liberate chlorine from HCl, which is distilled into KI. The iodine is titrated with thiosulphate.	Scott and Furman, p. 793.
Silver.	Titration with standard thiocyanate.	See p. 180.
Sodium.	Precipitation as pyroantimonate and titration of iodine after addition of potassium iodide.	Pincussen, W., "Mikromethodik" (Leipzig, 1925), p. 75.
Sulphur.	Oxidation to sulphate. Add excess of barium chloride; titrate excess of barium with standard chromate, using diphenylcarbazide indicator.	Compare Roth, H., *Z. angew. Chem.*, 1926, **39**, 1599.
Uranium.	Reduction to uranous salt and then titration with permanganate.	Furman et al., *J. Amer. Chem. Soc.*, 1932, **54**, 1344.
Zinc.	Titration with $K_3Fe(CN)_6$, using *o*-dianisidine as indicator.	Frost, A. F., *Analyst*, 1943, **68**, 51.

PART IV

COLORIMETRIC ANALYSIS

By R. F. Milton

COLORIMETRIC PROCEDURES

PART IV

COLORIMETRIC ANALYSIS

PRINCIPLES AND SCOPE OF COLORIMETRY

ALTHOUGH the micro-gravimetric and micro-volumetric methods of analysis described in Parts I and III are very wide in scope, suitable quantitative reactions cannot always be found for the exact analysis of every known substance, particularly in the fields of organic and bio-chemistry. Moreover, the limit of accurate micro-analysis by these methods is not always sufficiently low for technical requirements, particularly in clinical bio-chemistry, where only very small quantities of materials may be available for examination.

In these circumstances **colorimetric analysis** can often be used with advantage. In general its sensitivity for the estimation of metallic (and some other) ions is at least ten times that of the most refined micro-volumetric procedures and a hundred times greater than that of any gravi-metric method. In the analysis of organic substances colorimetric technique is as yet unequalled in scope as a method for the estimation of specific radicals or even of specific molecules (witness, for example, its use in the estimation of specific vitamins). Moreover, colorimetric methods are particularly attractive to the micro-analyst in view of their rapidity. In the main they involve the use of very little apparatus and bench space, and are often eminently suitable for mass-analysis, i.e. for the rapid estimation of series of routine samples.

Colorimetry may be defined as the process of measuring the concentration of a substance by making use of the fact that, on the addition of certain reagents, a coloured solution is produced in which the depth of colour is a simple function of the concentration of the substance to be analysed. Consequently a solution of unknown concentration can be estimated by comparing its colour with that of a similar standard solution made up, by weight, from known pure chemicals.

The original basis of colorimetry was the assumption that the visual intensity of "colour" of a solution is directly proportional to the weight concentration of the substance to be measured. With the advent of instruments which measure optical density, it is possible to estimate accurately, in light of regulated wavelength, solutions with which visual colour matching is impossible.

The theoretical principles of colorimetry can be summarised as follows:

If a beam of light passes through a liquid, then some absorption of radiant energy occurs, and this absorption will be increased as the depth of liquid

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increases. The absorption of visual light by pure water is so very small that if an aqueous liquid is coloured by a substance in solution then the light absorption is almost entirely due to the solute, the effect of the water itself being infinitesimal. The same is true for most other common solvents.

When viewed in transmitted light a solution exhibits a particular colour whenever the absorption of visible radiation is much less in one part of the spectrum than elsewhere. Energy due only to the non-absorbed wavelengths passes through the solution to be detected by the eye.

Experiments with monochromatic light have shown that if a beam of light of intensity I falls on a solution, then the amount dI which is absorbed in passing through a minute layer of liquid dl is proportional to the intensity of the light.

Consequently
$$\frac{-dI}{dl}=k'.I$$

If this equation be integrated between the limits I_o, the light entering the solution, and I_t, the light transmitted, then

$-\log_e \dfrac{I_t}{I_o}=K'.l$ where l is the total thickness of the absorbing solution.

$\dfrac{I_t}{I_o}$, the fraction of light which emerges, is usually called the *Transmittance*,

$=T$. The above equation may thus be written:
$$-\log T_T=K'.l$$
and in this form is known as **Lambert's Law.**

Beer's Law, which has also been verified experimentally for many substances, states that the absorption of light in a layer of liquid dl is proportional to the number of absorbing particles in this layer, that is to say upon the *weight concentration, c,* of the absorbent in the solution. Thus

$\dfrac{-dI}{dl}=k.c.I$ and consequently $-\log T=K.c.l$, where the constant K depends, of course, on the wavelength λ of the light concerned, so that, in more precise form, Beer's Law should be written as:
$$-\log T_\lambda = K_\lambda.c.l$$

Application of Beer's Law to the Colorimeter. It is obvious that k_λ (the absorption constant) is the same for any two solutions of the same substance, though their concentrations may be different. If two solutions are compared in a colorimeter and a match is obtained, then the transmittances (T) from the two depths of liquid must be equal. If l_1 and c_1 represent the depth of liquid and concentration of solute in the one case and l_2 and c_2 the corresponding quantities in the other, then

$$-\log T =l_1 c_1 k_\lambda = l_2 c_2 k_\lambda$$

Hence
$$l_1 c_1 = l_2 c_2 \quad \text{or} \quad c_1/c_2 = l_2/l_1$$

Thus when a colour match is made between two solutions the concentration of solute is inversely proportional to the depth of liquid through which the light is passing.

Validity of Beer's Law. Beer's Law, strictly speaking, refers only to radiation of any one particular wavelength. It is valid for the absorption of white light only for substances which have a comparatively simple absorption spectrum. Substances which have absorption bands in different parts of the spectrum may exhibit *dichromatism*: in this case the apparent colour of a solution is a function not only of the concentration of absorbing substance but varies with the depth of solution observed. Thus dichromate solutions appear yellow in dilute and orange in more concentrated solution. Certain pH indicators exhibit this phenomenon to a marked degree. Solutions of this type cannot be compared visually unless their concentrations are closely similar; but the difficulty of dichromatism may be overcome by using a source of light of narrow wavelength band or one from which the interfering colour has been filtered out.

Naturally Beer's Law is only valid if the basic assumption is true—that the light absorption is directly proportional to the *weight concentration* of the solute, irrespective of its dilution. In theory, therefore, Beer's Law is not exact for substances which ionise, dissociate, or form loose solvate complexes, but in actual practice it can be applied to most coloured substances, such as inorganic salts. According to modern concepts these are usually ionised, though not dissociated, even in the solid state, and retain throughout their water of hydration. Thus the characteristic blue colour of hydrated cupric ions is retained in crystalline copper sulphate. Slight effects due to ion distortion have been recognised, but in normal colorimetric procedures the possible experimental errors due to defects in tint comparison are far greater than those due to deviations from Beer's Law.

It is obvious that a colorimetric method may be used with safety for the analysis of a solution if its colour is due only to the component which is to be estimated. If there is present in the solution some other substance which also produces a colour with the same reagents, then the total depth of colour produced will not be proportional to the concentration of the substance to be analysed. In the same way colorimetric estimations cannot be made on solutions which are initially coloured, although this difficulty may often be overcome by the interpolation in the optical system of the measuring instrument of a light filter which counteracts the effect of the basic colour. For example, an initially yellow solution in which a blue colour is developed appears green to the eye. If, however, a yellow screen (of the same light absorption characteristics as the yellow solution) is put in the eyepiece, then the subsequent colour change may be taken as a measure of the blue product which is to be analysed. The standard, of course, must also be measured using the same light filter. Addition to the standard of

comparison of an equal amount of basic colour affords another way in which the difficulties due to initially coloured solutions may be overcome.

When colorimetric comparisons are to be made it is essential that the standard and the solution to be measured (the unknown) should have been prepared in an identical manner and that no turbidity should exist in either. It is impossible to compare two solutions if one of them contains a slight precipitate, or turbidity. Nevertheless, an accurate comparison of coloured turbidities may be made providing that the pre-requisites of accurate nephelometric technique described on pp. 269–273 are adhered to.

Methods of Colour Measurement

A number of different experimental techniques have been developed for comparative colour measurement. The particular method that should be used is dependent upon the conditions of analysis. If accuracy is the only criterion, then an objective type of instrument, such as the Spekker photoelectric absorptiometer (pp. 247-249) is to be preferred. Quite useful accuracy may be obtained with visual photometers, of which the Pulfrich instrument (p. 242) is an example. Convenience and speed may be the guiding need, in which case some of the less accurate procedures using quite simple and inexpensive apparatus can be utilised. The available principles or methods are as follows:

1. *Visual Comparison against a Series of Standards.* In this method the sample to be analysed is placed in a definite volume of liquid in a standard-size test-tube and the characteristic colour is developed by a chemical reaction. At the same time a series of standard solutions of the substance to be estimated is made up in identical test-tubes, each containing the same volume of liquid, and the colour is also developed in these solutions. The unknown sample is then compared visually with the standard colours until a match is agreed upon; the concentration of the selected standard then corresponds to that of the unknown solution. This method of visual comparison is widely used in water analysis, etc., and may be made more accurate if used in conjunction with a "comparator" or some such device (see p. 236–237). Permanent standards are often used: reference solutions are prepared and kept in sealed tubes or solid standards are made from tinted glass. In using this technique it is obvious that the range of the standards must overlap the concentration of the substance to be measured, so that comparison against both lighter and darker colours may be made. Also the differences between the successive standard colours must be small enough to give a good degree of accuracy of estimation. This last point is usually assured by first making a series of standards with gross concentration differences, and so gauging the position of the unknown, and then preparing a second series of standards graded with much smaller concentration differences and so pin-pointing the exact match of the unknown.

2. *Visual Comparison by Dilution.* In this method two tubes of identical bore are used to contain (*a*) the standard and (*b*) the unknown, and the characteristic colour is developed in each tube. The liquids in the tubes are compared by looking *through the thickness of the tube* (not through the thickness of the liquid from above). The liquid in the darker tube is then diluted with distilled water and mixed. This dilution process is continued until the two tubes when viewed from the side are identical in colour. When equal volumes in each tube exhibit the same amount of colour they must contain equal concentrations of the coloured solute, and consequently the total amounts of the substances in the two tubes will be directly proportional to the respective total volumes of solution.

This method is often very convenient, but may be of doubtful accuracy in some cases. Very often the amount of colour produced by a reaction—although basically proportional to the concentration of the substance to be estimated—may be grossly affected by the concentrations of the other reagents added to produce the colour. An instance of this is given in the colorimetric estimation of phosphorus by the ceruleo-molybdic method (see p. 314), wherein very rigid adherence to the correct concentration of sulphuric acid is required in order to obtain a colour directly proportional to the concentration of phosphate.

3. *Addition of the Standard until the Colours are Identical (Balancing).* Two tubes of identical diameter are set up. Into one is placed the unknown together with the necessary reagents to produce the appropriate colour. To the other tube is added the colour-producing reagents, and then a standard solution is titrated in, drop by drop, until the colours in the two tubes appear identical when viewed *from above.* Then since the colours in the total liquids are identical, the tubes must contain identical amounts of the substance to be analysed. The concentration of the unknown solution is thus equal to that of the measured volume of standard solution added.

This procedure may suffer from the same defect as mentioned under 2, namely, that the two tubes may not contain identical volumes of liquid and consequently the concentrations of the reagents may likewise vary, with possible effect upon the colour.

The following modification of the above method to a large extent overcomes this disadvantage. Two identical tubes are put up and the colour is developed in the unknown solution. To the other tube is then added the same volume of colour-producing reagent and also distilled water until the volumes in the two tubes are almost identical. Strong standard solution is then run into this tube from a micro-burette until the colour match is obtained. By adopting this procedure errors due to volume differences are obviated.

4. *Balancing by Variation in the Depth of the Liquid.* In this method the depth of the liquid is increased or decreased until, when viewed through its

depth, the colour in the unknown solution is identical with that of a standard. The concentration is then inversely proportional to the depth of the liquid. This procedure may be carried out by adding or subtracting fluid from the tube containing the substance to be estimated, or it may be done by projecting into the liquid a glass tube connected to an eyepiece until the colour, as seen through the tube, is identical with that of the standard. The concentration of the substance will then be inversely proportional to the depth of liquid below the level of the viewing tube. This in effect is the principle of the Duboscq colorimeter (pp. 240–241).

It is the method most commonly used and the most accurate procedure in visual colorimetry.

5. *Comparison against the Thickness of a Coloured Wedge or Variable Aperture.* The solution to be measured is compared in its depth against a standard wedge of the same tint. By altering the position of the wedge a match is made. The reading on the wedge may be calibrated against standard solutions and so the concentration of the unknown substance can be estimated.

This principle is used in the *photometer* type of instrument, in which a neutral wedge or variable aperture is interpolated in one side of the optical system and the unknown solution in the other. The light beams are usually brought together in an eyepiece into which is inserted a filter giving a narrow wave-band, so making for simplicity of comparison. These photometers are undoubtedly the most accurate of the visual instruments.

6. *Measurement of the Light Falling upon a Photo-cell.* Because of the variability of perception of the human eye in matching light intensities, the need for an objective type of instrument arose. This was possible with the development of the light-sensitive or photo cell. The latter comprises a layer of a sensitive substance, which, under the action of light, becomes activated and generates a small current proportional to the amount of radiation falling upon it. This current may be measured on a microammeter.

In its simplest form the instrument consists of a constant source of light, a glass cup or cell to contain the liquid to be measured, and a photo-cell. A galvanometer reading is taken with distilled water in the cup and then a second reading with the solution to be measured. The difference in galvanometer readings is proportional to the radiation absorbed by the solution. If this procedure is followed, then by using a series of standard solutions a curve relating galvanometer reading to concentration may be constructed.

There are many disadvantages to this simple type of apparatus (see p. 244), and in consequence it is better to use a null-point type of instrument in which two photo-cells are coupled up so that no current is flowing when the intensities of the light falling on the two cells are identical. A common source of light is split, one part passing through the unknown solution and

the other through a graduated wedge or calibrated light-extinction device of some kind. Variation of the latter is made until the galvanometer reading is zero, thus indicating that the light passing through the unknown solution is equal to that passing through the wedge. Calibration of the instrument by using a series of standard solutions is made in the usual manner.

Instruments of this kind, if used intelligently, are by far the most convenient and give the most accurate results. Of course, only total transmitted energy is recorded, and any variation in colour tone or the presence of turbidity could pass unnoticed and lead to inaccuracy of results. It is essential, therefore, that a visual inspection be made before each analysis to ensure that the colour of the unknown is identical in tone with the standard and that both solutions are absolutely clear.

ACCURACY OF COLORIMETRIC ANALYSIS

THE choice of a colorimetric procedure for the estimation of a substance is dependent upon a number of considerations, e.g.:

(i) A colorimetric method is often chosen because no satisfactory gravimetric or volumetric procedure can be applied, and this is particularly so in the estimation of biological substances.

(ii) A colorimetric method will often give a greater degree of accuracy in the low limits of micro work than the corresponding gravimetric or volumetric process.

(iii) A colorimetric method is frequently chosen because of a greater degree of specificity.

(iv) The use of colour methods is being increasingly applied because of the rapidity with which the estimations may be carried out. This is especially so in mass analysis and in the routine estimation of a number of samples. In the latter case, extreme accuracy is often sacrificed for speed, and for this reason there still exists in the minds of many analysts of the older school a prejudice against all colour methods. It cannot be over-emphasised that colour methods, if rigidly controlled, may give a degree of accuracy corresponding with the limits of the best micro-volumetric and gravimetric procedures, and often on infinitesimal quantities of substance.

CAUSES OF ERROR IN COLORIMETRIC ANALYSIS

It is essential to have an appreciative knowledge of the possible causes of inaccuracy in colorimetric analysis. These will, of course, depend on the conditions applying to the particular estimation, but may nevertheless be summarised in the following table.

TABLE IV.1

SOURCES OF ERROR IN COLORIMETRIC ANALYSIS

I. *Chemical Sources of Error*
   (a) The effect of substances other than the "unknown" present in the solution.
   (b) Variations in colour intensity due to the addition of varying amounts of reagents.
   (c) Errors resulting from incomplete development of colour.
   (d) Errors caused by fading of colour.
   (e) The effect of temperature.
   (f) The effect of time of standing.
   (g) A tendency for coloured products to precipitate.
   (h) Differences in refractive effects due to variations in sizes of colloidal particles.
   (i) Use of substances having colours which are not completely proportional to concentration.

II. *Mechanical Causes of Error*
    (*a*) Mechanical faults in the measuring instrument.
    (*b*) Errors due to optical effects in the solutions.
    (*c*) Errors due to variation in quality and quantity of light source.
    (*d*) Faults in the optical balance of the instrument.
    (*e*) Subjective errors due to the operator.

III. *Errors Due to Faulty Technique*
    (*a*) Inaccuracy or oversight in the measurement of the reagents to be added
        or in the extent of the dilution.
    (*b*) Inadequate mixing of the solutions used.

It is an advantage to consider these possible sources of error with some degree of thoroughness. **Errors due to chemical causes** are the chief possible inaccuracy. The *presence of substances other than the unknown* may modify the colour in a variety of ways. If, for instance, a colour reaction is not specific or is specific for a group of substances, then complications arise when other members of the group are present. As an example, the estimation of benzene after nitration and action with butanone and alkali can be cited (see p. 342): toluene and xylene also give colours, and these, if present in small quantities, will cause inaccuracy in the assessment of the benzene concentration.

Some substances may effect a colour reaction by forming complexes with the substance to be estimated and so prevent ionisation and consequent chemical reaction. Thus the presence of fluorides invalidates the colour reactions with a number of metals. Pyrophosphates may have a similar effect.

Varying amounts of reagent may affect colour production. High concentrations of reactants may alter the mass action equilibrium position. Thus in the presence of large amounts of ammonium salts, the back-reaction in the ferric thiocyanate equilibrium $FeCl_3 + 3NH_4CNS \rightleftharpoons 3NH_4Cl + Fe(CNS)_3$ is increased. This leads to low results being obtained. For the same reason a large excess of thiocyanate will push the reaction nearer to completion.

Variations in concentrations of organic solvents may affect colours. Colour variations with certain dyestuffs have been ascribed to alterations in the resonance state of the substance due to differences in solvation. Amounts of acid and nature of acid may affect a colour intensity, particularly if the coloured product is an indicator, and in this case salt effects may also modify the result (see p. 257).

Often a colour takes some time to develop completely. It follows that a colour reaction of this type may be incomplete and hence not comparable with a standard solution unless both samples have been prepared under identical time conditions. Of course, the presence of other solutes may modify the development time, and this should be borne in mind when preparing the standard colour.

In the same way *a colour may reach its peak and then begin to fade.* It is essential then to measure its intensity at the peak of the time-development curve.

Colour development is often enhanced with increase in temperature, but similarly the rate of fading is also speeded up. Unless standard and unknown are prepared under identical conditions with regard to temperature, then faulty results may occur. Depth of colour and "tone" may also be affected by temperature. Again, with dyestuffs the effect may possibly be due to intra-molecular resonance phenomena.

In some cases where the colour is really due to a colloidal solution of a substance (e.g. copper ferrocyanide in the estimation of copper), there is a tendency for the colour to be modified according to the exact experimental conditions, for example the temperature, the "age" of the preparation, etc. Colloids such as $Cu_2Fe(CN)_6$ are susceptible to "salt effects," and the presence of other solutes may cause rapid coagulation and precipitation.

To some extent these difficulties may be overcome by the use of a protective colloid such as gum ghatti (see p. 266), but in coloured solutions of this type differences in refractive index of the solution may occur in consequence of changes in degrees of hydration or of dispersion of the colloid substance. In such instances Beer's Law (p. 225) is not followed and the strict mathematical considerations of colorimetry do not apply.

Refractive index difference may also be significant if the solvents used in the two compared solutions are not identical in concentration.

These points emphasise the need to ensure that standard and unknown solutions should be as identical as possible in all respects, including concentration. Where a series of estimations are to be made over widely differing concentration ranges, then possible errors due to deviations from the linear relationship between colour and concentration may occur. This error may to a large extent be overcome by utilising a series of standards of range similar to that of the concentration to be measured.

The chief **errors due to mechanical causes** are traceable to faults in the instrument, in its usage, or to lack of attention to operational details. For instance, it is essential that the measuring instrument be correctly calibrated. In a visual colorimeter the scales should be linear, accurate, and comparable with one another. The optical system should be balanced; and where prisms are used, then the arrangement should, at frequent intervals, be checked carefully for alignment, since in order to obtain correct matching of the colours it is essential to ensure that the quantity of light entering both solutions is identical. Uneven light absorption in the instrument should be avoided. Thus the cups used for holding the solutions should be identical in character, particularly with regard to the thicknesses of the bottom plates. For the same reason the eyepieces of instruments should

be kept free from dust. This applies also to screens, condensers, reflectors, etc.

A frequent source of error is a dirty cup or comparison cell. This is often the result of repeated use of the instrument with colloidal suspensions, which eventually leave a slight deposit on the sides of the cell. Such a film may have a significant absorption effect and result in faulty comparison, although the contaminant may scarcely be visible to the naked eye. When a liquid to be measured is poured into a comparison cell, care should be taken that the fluid does not spill on to the sides or bottom, since this extra film of liquid in the light path causes an error of reading. Also cups and cells may become etched or scratched, and so cause uneven light transmission. In photo-electric instruments the light should pass through a standard thickness of liquid and consequently the thickness of every cell (and of its glass sides) should be the same.

With Duboscq colorimeters, uneven or loose positioning of the plungers is a possible source of error, but this fault, and also those due to faulty optical alignment, may be checked by reading the instrument a second time with the cups changed into the opposite holders. Identical readings should be given if all the points enumerated above are correct.

The *quality of the light* used when making colorimetric comparisons may have a considerable bearing on the accuracy of the results. In general, direct light should be avoided, and for ease of comparison diffused daylight is the best. Some colours, e.g. yellows, are more accurately compared under specific light conditions, and, with diffused daylight as the source of illumination, better results are given on bright, sunny days than on dull, cloudy occasions. Because of the inability to control the constancy of diffused daylight, an artificial source of light, preferably that from a "daylight lamp," is advocated for routine work. The lamp should be arranged so that the light is evenly distributed into the optical routes of the measuring instrument after being reflected and diffused by impingement upon an opaque glass plate at an angle of about 45°.

Apart from the errors due to optical effects mentioned above, there occur occasionally errors due to dichromatism, which has been mentioned on p. 226, and which may be overcome by using as the source of light a waveband from which the interfering colour has been filtered out.

All visual colorimetry suffers from the drawback of being subjective to the efficiency of the observer. Colour-blindness is much more common than is generally realised, and the ability of individuals to match different colours varies enormously. In general, the matching of colours by eye is accurate to about 5%, but with a colorimeter this may, after considerable practice, be reduced to 1%. The accuracy varies not only with the individual but also with the colour which is being measured and with the quality of the light used for illumination. Some individuals can only compare with one

eye, and it is rare to find that both eyes give an identical match. Eye-strain and fatigue is another factor that causes much trouble. After practice, best results are obtained when the matching is made rapidly. Peering through an eyepiece for long periods makes colour matching very uncertain. Green and blue colours are most easily compared, yellow and red are the most difficult and cause fatigue. The accuracy of reading may be enhanced if a number of comparisons are made at different dilution against an appropriate standard.

Errors due to faulty technique on the part of the operator are not specifically errors of colorimetric analysis. Nevertheless, in methods where micro quantities are being measured, especial care must be taken to ensure that contamination is avoided, that weighing is accurate to the limits required by the method, and that dilution is carried out correctly. Thus, in the method for the estimation of phosphate (p. 314), a standard solution is made by weighing out a quantity of potassium di-hydrogen phosphate. This is then dissolved in 1 l. of water and 10 ml. of this solution again diluted to 1 l. A further dilution is subsequently made. Consequently, an incomplete mixing in the first solution will give rise to a considerable error in the final concentration, and to a lesser extent the same will be true if the mixing were defective in any of the subsequent dilutions.

A dilution error will also occur if the colorimetric tube is not dry. Again in serial determinations, if a comparison of a concentrated colour with a standard is followed by an examination of a more dilute colour, then it is essential to rinse the cup out with a little of the dilute solution before filling it to make the comparison with the corresponding dilute standard.

When dealing with artificial standards for comparison, errors of dilution may result due to the dichromatism phenomena mentioned above, and these points must be borne in mind by the observer.

APPARATUS

THE apparatus associated with colorimetry varies from simplicity to extreme complexity. The simplest form consists of a series of test-tubes and the most complex are the latest types of photo-electric instruments. The general technical developments of the various instruments have aimed at attaining greater accuracy in measurement, first by diminishing the possibility of optical error by refinement in ocular instruments, and then by replacing the human eye by the photo-electric cell and so making the measurement entirely objective.

A. VISUAL INSTRUMENTS

1. The Comparator

In its simplest form the comparator consists of a series of test-tubes containing a range of standard solutions of the substance to be estimated. The unknown is also contained in a similar test-tube and in a similar volume of liquid. Then, by making a visual comparison, the position of the unknown is ascertained in the standard series. Sometimes it is necessary to prepare two sets of standard solutions, one to obtain the approximate concentration of the unknown and then a second series with much smaller intervals than the first in order to pin-point the value of the unknown.

The Nessler method is a variation of the above. It requires a set of about a dozen cylindrical glass tubes ("Nessler tubes"), of equal diameter and with plane polished bottoms, having graduations at 50 and 100 ml.

A series of standard solutions are placed in Nessler tubes and are then made up to the appropriate volume (50 ml.) with reagents and distilled water. The unknown coloured solution is prepared and diluted to the standard volume, and its tint is then compared with those of the standard tubes until a match is obtained. *Viewing is, however, made from the top through the depth of the liquid,* and is best carried out by placing two tubes side by side on a white tile and in a good diffused daylight. By using such standard tubes in the manner described above, possible errors involved in the use of test-tubes, due to uneven thicknesses and variations in curvature, may be obviated and a quite accurate match can be made. Moreover, by viewing through the depth of the liquid (over 10 cm.) instead of through the thickness of the tube (1–2 cm.), a higher degree of sensitivity may be obtained.

The comparator method of colour measurement may be used quite effectively to estimate solutions which have a natural background colour.

In this case the viewing is made through the thickness of the liquid and it is best to use some form of apparatus such as that elaborated by Mines* for pH measurement. This consists of a block cut to take two sets of tubes in line, and the block is symmetrically pierced so that a beam of light will pass through each of the two sets of tubes (see fig. IV.1).
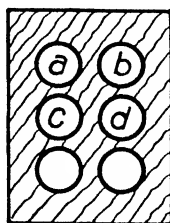
In tube a is placed the coloured solution to be estimated, to which is added the appropriate reagents to produce the correct colour reactions. In tube c, which lies directly behind a, is placed distilled water. Into the first tube b in the second row is placed the raw coloured solution without any reagents, and into tube d is put a standard solution of the substance to be estimated together with the neces-
sary colour producing reagents. a and c are viewed in line and b and d also. The standard solution in d is changed until the combination $(a+c)$ matches exactly $(b+d)$. In this way the natural colour of solutions may be allowed for.

When using the test-tube technique it is often convenient to have permanent standards for that purpose. The best pro-cedure is to seal the standard colour in the tubes. Such sets of standards may be obtained commercially for pH work.

There are several types of appa-ratus for use as comparators, for instance the *Roulette pH com-parator*, in which the standards are made to revolve past the un-known; and the *Coolidge appa-ratus*, wherein the standards are

Fig. IV.1.   Mines' Comparator.

arranged in a rack which slides past the unknown and so makes matching more simple.

Comparators are most useful for carrying out rapid determinations where a tone range of colour is encountered, such as in pH work.

Many liquid colour standards do not keep indefinitely, though their deterioration may not be evident after many months. Organic dyes may fade due to photo-chemical decomposition, whilst hydrolysis of the glass may affect pH indicators. Substitute standards containing salts of metals such as chromium, copper, cobalt, or nickel have been used with advantage on account of their permanency.

* Mines, G. R., *J. Physiol.*, 1910, **40**. 327.

2. The Lovibond Tintometer

This apparatus involves the use of solid glass standards in place of liquids. In the earliest form, the tintometer consists simply of an arrangement for comparing the colour of a liquid against a number of coloured glasses without any optical system except an eyepiece to ensure that parallel rays of light reach the eyes. The later models make use of artificial light reflected from an opaque white screen (fig. IV.2). The apparatus has had very wide application, particularly in fields where absolute estimation is impossible, such as in the colour-testing of natural waters, oils, caramel, spirit, wines, and dyes. In each case a series of coloured glasses of graduated depth are used and the result is expressed in "Lovibond units." These glasses are quite permanent and are standard with the instrument. The modified form for use in pH determinations is similar to the comparator, but contains a disc into which is fitted round the periphery the permanent glass standards.
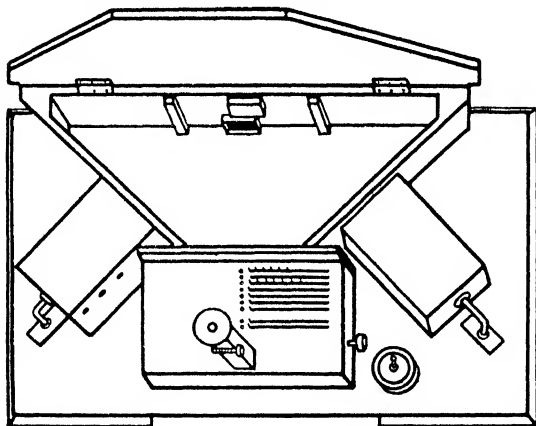
Fig. IV.2. Lovibond Tintometer.

The disc may be revolved to bring the appropriate colour in front of the blank test-tube, whereby it may be compared directly with the unknown. This model has also been adapted for numerous other determinations such as the estimation of chlorine in water by the o-tolidine method (see p. 309).

The *Hellige comparator* is similar in principle to the Lovibond instrument, but it incorporates a much more refined optical system (see fig. IV.3). Light from a constant source is brought through semi-opaque glass, and passes in two parallel beams through similar tubes containing water a and the unknown solution b. A disc with coloured glasses is arranged so as to revolve in front of a. The two beams of light are then brought side by side by refraction through a double Helmholtz prism into the eyepiece where comparison is made.

The Lovibond tintometer is particularly useful when assessing natural colours, but for exact quantitative work should, to a large extent, be

superseded by the visual photometers (which measure in absolute units) or the photo-electric instruments (see pp. 244–249).

3. The Dilution Colorimeter

In its simple form this consists of a box with two holes into which are placed graduated tubes. A slit through the box, low down, allows light to pass horizontally through the two tubes containing the solution and the standard respectively, allowing comparison to be made. In operation the unknown solution is contained in a certain volume and the standard (which
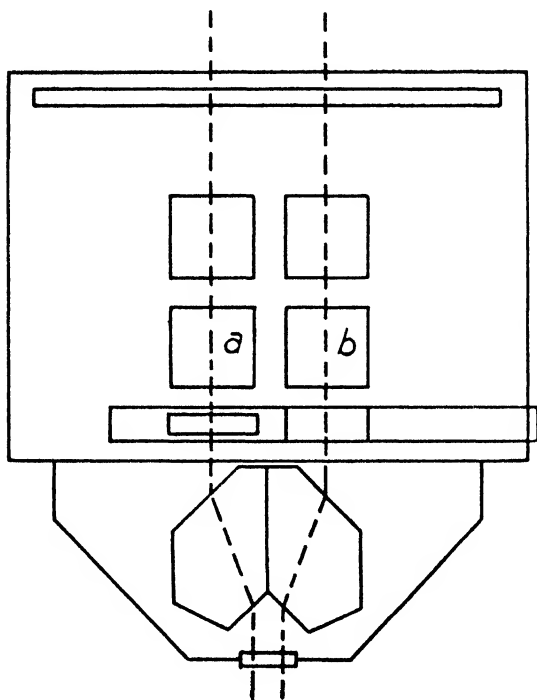
Fig. IV.3.   Diagram of Hellige Comparator.

is usually the more concentrated) in a similar volume. Water is then added to the standard and after mixing is compared with the unknown, this dilution process being carried out until a match is obtained. Then the concentration of the two solutions per unit volume is identical and from the volumes the concentration of the unknown may be obtained. This apparatus may also be used in the reverse manner. Thus the unknown colour is contained in a given volume in one tube and the colour-producing reagents in a similar volume in the second. Standard solution is then added, drop by drop, from a micro-burette to the second tube until the colours match. Again concentration is proportional to volume.

The dilution colorimeter is only of value when the colour is produced immediately on addition of the solutions and when variation in the dilution of reagents in the solutions has no effect upon the colour subsequently produced.

4. Balancing Colorimeters

A. Nessler Tubes. A pair of Nessler tubes, as mentioned on p. 236, may be used to make colorimetric comparison by the balancing method. The two tubes containing equal volumes of the colour-producing reagent are placed side by side on a white tile. The unknown solution is pipetted into one tube, and to the other is added, from a burette, the standard solution until the two tubes, when viewed vertically through the depths of liquid, are identically coloured. The amount added from the burette is then measured.

B. The Kennicott Colorimeter. This consists of two tubes of similar diameter, and with plane bottoms, placed in position so that parallel light passes upwards through the length of the tubes after reflection from an opaque glass. The light is then reflected from mirrors into an eyepiece in such a manner that a comparison between the two tubes may be made.

One of the tubes is fitted near its base with a side-arm connection to a cylindrical reservoir, which is fitted with a glass plunger. To operate, the unknown solution is placed in one tube and the standard colour in the reservoir of the other. By means of the plunger, solution is forced from the reservoir into the second tube until the two tubes, when viewed through the eyepiece, show a match. Then the concentrations of solutes in the two liquids will be proportional to their respective heights.

C. The Duboscq Colorimeter. The logical development of the colorimeter described in the previous paragraph is the Duboscq type of instrument. Various modifications of this have had very wide applications during the past twenty-five years. It is very simple to use, matching is quite accurate, and a result is obtainable with only a few millilitres of liquid.

The Duboscq colorimeter is similar to the Kennicott model in that it relies on an alteration in the depth of the liquid column to achieve balancing, but differs in so far as it increases or decreases the length of column by means of a glass plunger.

The apparatus consists of a pair of cylindrical plungers of solid glass fitted to an optical arrangement whereby, by means of prisms, the light passing through the plungers is brought into an eyepiece, thus giving side by side two semi-circular fields of illumination. Two glass cups of equal diameter and having plane glass transparent bottoms, stand upon two movable holders. These holders are pierced by a small hole to allow the passage of light through the bottoms of the cups and up through the liquid. By means of a knurled knob, the holders may be moved independently

up or down on ratchets, and this allows the cups to be moved towards or away from the plungers, which are so arranged that they lie in the vertical axis of the cups. At the bottom of the instrument is a milk-white plate placed on a swivel so that light from a horizontal source may be reflected vertically up through (i) the holes in the stands, (ii) the bottom of the cups, (iii) the liquid in the cups, and (iv) the glass plungers, and so to the eye-piece (see fig. IV.4).

The length of the columns of liquid between the bottoms of the plungers and the bottoms of the cups may be increased or decreased by raising or lowering the stands. Attached to the ratchets operating the stands are two graduated scales. In operation, the unknown liquid is placed in one cup and the standard in the other. The standard is set at a given depth and the unknown is raised or lowered until a match is obtained. The respective concentrations are then inversely proportional to the depths of liquid between plungers and cup bottoms, and this is indicated by the scale settings.

The Duboscq type colorimeter has many variations, but is, without doubt, the most accurate of the visual instruments for making direct comparison. The *micro instrument* is designed by a direct scaling-down of the usual instrument. The cups used have a capacity of 1 ml. and are thus eminently suitable for biological work, where only small quantities of liquid are obtainable.

Fig. IV.4. Diagram of Duboscq Colorimeter.

Where artificial light is used in conjunction with the Duboscq model, care should be taken to ensure that the two sides of the apparatus are evenly illuminated. The most convenient method of securing this is by inserting a condenser system between the source of light and the reflector plate.

The balancing colorimeter may be used in almost any circumstances where Beer's Law is followed and where no turbidity concomitantly exists.

5. The Visual Photometer

Strictly speaking, a photometer is an instrument to measure light intensity. The type of photometer used in colorimetry allows of measurement, in absolute terms, of light transmitted through a coloured liquid. Photometers in this sense may be classed with instruments using permanent glass standards, since the principle involved consists of balancing the light passing through a liquid with that passing through a graduated wedge of darkened glass or through a calibrated iris diaphragm.

The best known instrument of this type is the *Pulfrich step photometer* (see fig. IV.5). In the vertical form this is arranged rather like a colorimeter in that there are two cups with transparent bottoms, and above these two telescopic systems which unite the transmitted light by a prism system into an eyepiece as two halves of the circle. Light is reflected off a swivel mirror in two parallel beams up through the cups and the optical system to the eyepiece. In front of the eyepiece is a slot for the insertion of coloured filters, to obtain light as nearly monochromatic as possible. In place of the plunger system, an iris diaphragm is fitted to the ends of the telescopes above the cups, so that the total amount of light entering at each side of the optical system may be varied at will.
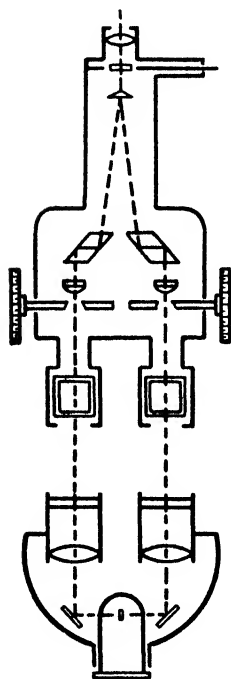
Fig. IV.5.
Diagram of Pulfrich
Step Photometer.

Into one cup is put the coloured solution to be measured and into the other distilled water (or the solution without the colour-producing reagents). The instrument is then fitted with the appropriate colour-filter, or "screen," to give a narrow band of transmission complementary to the colour to be measured, and the iris on the blank side of the instrument is stopped down until an optical match is obtained in the eyepiece. The absorption of light by the unknown is then equal to the amount of light cut off by the iris. Since this latter is calibrated, then the light absorption may be measured.

The same principle is used in visual spectrophotometers, with which accurate measurement can be made in monochromatic light.

The *Vernes-Bricq-Yvon* instrument utilises rather a different principle (fig. IV.6). A source of light is lensed to give a broad parallel beam of light, one half of which goes to a right-angle prism A and the other half on to prism B. The light falling upon A is totally reflected and after passing through the liquid to be measured, which is held in a solution-holder H—a glass cell with parallel sides exactly 1 cm. apart—falls on prism C, at which it is again totally reflected so as to reach one side of the double prism D.

D is a cube made from two right-angle prisms on the hypotenuse of which is silvered a strip of mirror *S*. The light proceeding from *C* passes through *D* except that which hits the back of the mirror strip *S*. This passes on to *E*, where again total reflection occurs, and so it goes to the eyepiece *F*, where it would appear as a disc of light with the centre strip darkened. The other half of the beam passes to prism *B*, where total reflection occurs, and then through a smoked graduated wedge *W* which may be moved across the path of the light at right-angles. The light then passes through cube *D*, except for that portion which hits the front of mirror strip *S*. This portion is then reflected at right-angles to prism *E* and eyepiece *F*, thus completing the circle of light therein.

By varying the position of wedge *W*, more or less light is brought through the balancing side of the optical system, until a match is obtained. Then
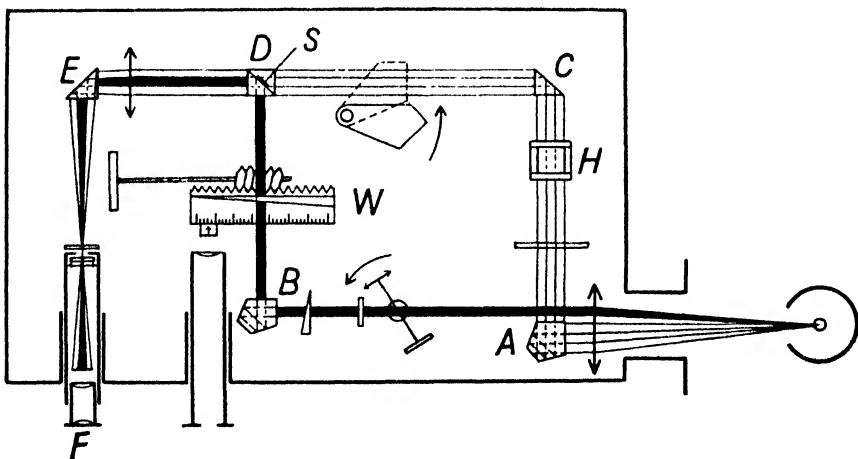
Fig. IV.6. Diagram of Vernes-Bricq-Yvon Photometer.

the amount of light absorbed by wedge *W* is equal to that absorbed by the solution in *H*.

The wedge is calibrated in terms of *optical density*, which is defined as *the ratio of light transmitted to light absorbed.*

Provision is made in this instrument for the insertion at the eyepiece of coloured filters, so that there is no apparent difference in colour tone in the two halves of the optical system when comparison is being made. *Wratten filters* provide a wide range of colour screens with narrow transmission bands, and so allow any coloured solution to be measured with this instrument in absolute units.

When using photometers in colorimetry, it is necessary to standardise the instrument against the particular substance to be estimated. This process (which is discussed more fully on p. 264) entails making a series of

standard colours and reading their optical densities on the instrument. A graph is then prepared relating optical density to concentration. To this, the reading given by an unknown solution may be subsequently referred. Photometers may also be used to measure turbidities, if precautions be taken to prepare exactly corresponding suspensions of standard solutions of the same substance (see pp. 269–273).

Photometers are the most accurate of all the visual instruments, for they allow of a very fine degree of light differentiation and refer always to a permanent standard for comparison. Since these instruments require only 1 ml. of liquid for comparison they are also suitable for ultra-micro work.

B. PHOTO-ELECTRIC INSTRUMENTS

General Considerations

The prime object of the photo-electric instrument is to replace the human eye by a less subjective form of measurement. The chief drawback in colorimetry is the difficulty of reproduction of readings by different observers, due to individual variations in colour perceptibility. This is particularly noticeable when orange and yellow colours are being observed, although it is to some extent less marked when an appropriate colour filter is incorporated in the colorimeter eyepiece. With the photo-electric instrument, however, the only visual observation is that of a galvanometer reading, and since this may be made with accuracy by all, colorimetry is removed from the realms of personal judgement to those of absolute mechanical measurement.

The earliest photo-electric instruments consisted of a source of light, a cup to contain the coloured liquid, a photo-cell, and a galvanometer. There were a number of disadvantages to this arrangement, and so development proceeded along lines to obviate the grosser defects. This "single cell instrument" depends upon the constancy of the light source for its reproducibility, and this, in practice, is relatively difficult to ensure. Since the galvanometer reading varies with the amount of light falling upon the photo-cell, it is easy to see that variations in the intensity of the light source will materially affect the readings. A second disadvantage to this arrangement is the tendency for some photo-cells to become "fatigued" when subjected to a continuous impingement of light waves, and so to produce a current which diminishes as time passes. This is manifest by a diminution in the galvanometer reading. A further point to be noted with instruments of this type is the fact that the ratio *light intensity/current produced* is usually linear only over a very small range of light intensities, and thus there is a tendency for lack of sensitivity to be experienced with more deeply coloured solutions.

These points are to some extent allowed for by the more modern types, of which the *Evelyn photometer* and the *Morris photometer* are examples.

The *null-point* type of instrument does not suffer to the same extent the disadvantages mentioned above.* Most null-point systems incorporate two photo-cells, arranged in electrical circuit so that no current is flowing in the galvanometer when the light falling upon them is identical. Hence, since the galvanometer reading always represents the difference in light transmission through two solutions, it is independent to a very large extent of any variations in the intensity of the source. In the same way, cell fatigue, due to continual light impingement, tends to be of the same order for both cells, and this makes readings independent of such phenomena. With regard to the third point—lack of linear photo response with low intensities—this becomes purely a matter of the accuracy with which the null-point may be reached, and a sensitive galvanometer will ensure this. On the other hand, if the two photo-cells have uneven spectral responses, then an inaccurate balance will result. For this reason the pairing of the cells should be done with great care.

Fig. IV.7.   Diagram of Evelyn Photometer.

1. The Direct Reading Instruments

A. The Evelyn Photometer† (fig. IV.7). This instrument, which is commercially available in U.S.A., consists of a source of light of very low intensity *a*, which may be controlled by a series of resistances. A test-tube absorption cup *b* is used (which allows the colour reaction to be performed in the same vessel as that in which the reading is made), followed by the photo-cell *c*, and a galvanometer attachment *d*. A series of filters *e* is supplied, to be fitted in front of the photo-cell for use with differently coloured solutions. This instrument overcomes the general objections to single cell instruments by using as light source a 6·2 volt flash-lamp bulb with a consumption of 200 milliamps. It can therefore be used in conjunction with a car battery and causes very little drain on the

* For a survey see Müller, R. H., *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 571; 1941, **13**, 667.
† Evelyn, K. A., and Capnaiv, A., *J. Biol. Chem.*, 1937, **117**, 365.

accumulator, thus ensuring an invariable source of light. The lamp is always used well below its rated voltage.

By means of resistances, the light is varied so that the galvanometer reads 100 when there is no absorption in the solution. The solution to be measured is then inserted in the instrument and the consequent galvanometer reading is thus an immediate measure of the percentage transmission. The galvanometer used has a main coil of 1,000 ohms and an outside compensation coil of 500 ohms. One hundred divisions on the galvanometer is equivalent to a current of about 2·5 microamps.

For each type of estimation a graph relating percentage transmission to concentration is prepared by submitting standard solutions to the measuring procedure.

B. *The Morris Photometer*[*] (fig. IV.8). This instrument has the following features, which overcome most of the objections to single cell instruments.

(*a*) A very sensitive photo-cell and galvanometer are incorporated. This enables a light source of very low intensity to be used and ensures that the low, or linear, part of the photo-cell response is utilised. Moreover, because of the fact that only a small amount of light falls on the photo-cell, the danger of fatigue is greatly diminished.

(*b*) A 6 watt 6 volt bulb is used as the source of light, and as this amperage may be safely taken from any 6 volt car battery, a steady light source is maintained, particularly if the accumulator is put on trickle charge when the instrument is not in use. Alternatively, by the use of constant-voltage transformers, the instrument may be operated with mains current. With two transformers cross-connected a very efficient smoothing
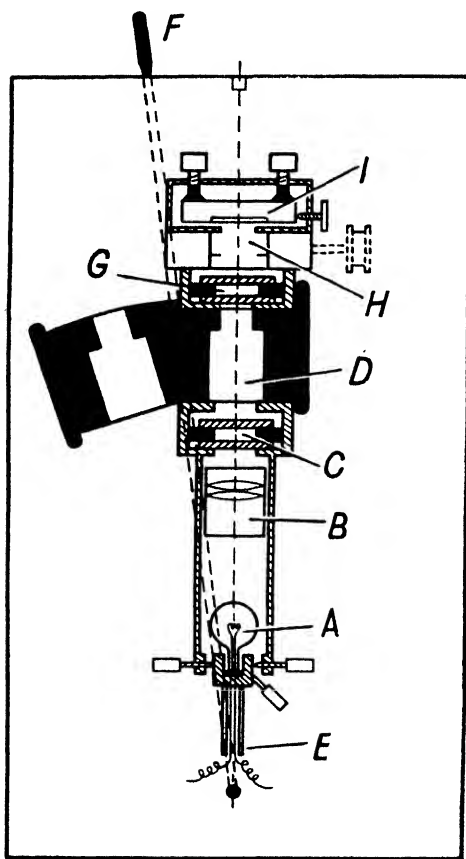
Fig. IV.8.   Diagram of Morris Photometer.

of mains fluctuation results, as an 8% change in mains voltage only produces a 0·2% deviation in the galvanometer reading.

The instrument consists of a light source A with electrical connections soldered directly to the lamp. A centring device is incorporated. B is an optical condenser, adjustable to give a parallel beam of light. C is a filter holder, to take two filters, one of which is an infra-red filter kept permanently in position. D is a liquid cell carriage with a 1 cm. optical stop to obviate effect due to reflection from the meniscus or sides of the cell. The carriage is built in the form of an arc pivotted at E, and is moved by lever F. When F is in mid-position, the light to the cell is interrupted and the filters may be changed without danger due to over-illumination. The carriage accommodates two optical cells to contain the liquid. G is a second filter holder and a range of Ilford spectrum filters is recommended to cover the visible spectrum. H is an iris diaphragm with worm-drive fine adjustment and I is the photo-cell. A Tinsley enclosed type galvanometer of coil resistance 150 ohms is used, thus ensuring linearity of reading over a wide range of illumination.

To use the instrument solvent is placed in one cup and the galvanometer scale adjusted to 100. The solution to be measured is then placed in the other cup and again the scale reading taken; this then represents percentage transmission.

2. Null-point Instruments

The Hilger "Spekker" Absorptiometer. This is the best known example of the balanced type or "null-point" instrument. In principle it consists of a pair of photo-cells linked up in a Wheatstone circuit. When the current generated by the two cells is identical, then a zero reading is shown on the galvanometer. There is a central source of light. Between this and the photo-cell on one side is a cup to contain liquid and an iris diaphragm to vary the light falling on the photo-cell. On the other side is a guillotine diaphragm operated by a graduated wheel (to reduce the light falling on the cell to a required amount), then a glass cell, followed by the photo-cell. An optical system is incorporated into both sides of the instrument, so arranged that the image of the filament falls upon the photo-cell. This ensures always that the illuminated area of the photo-cell remains constant. The calibrated wheel operating the light guillotine is arranged so that $R = \log a$, if R is the reading when $1/a$ of the total light is transmitted.

The apparatus is best understood by reference to the diagram (fig. IV.9). A is a lamp-housing covering a 100 watt lamp of triple filament. B is a cup of parallel faces and standard thickness. C is an iris diaphragm which regulates the light falling on the photo-cell D. On the other side of the lamp-housing is the guillotine E with its calibrated wheel, followed by a cup G (again standard) to hold the solution to be measured. Immediately in front of G is a lens (which causes a parallel beam to pass through the cup),

and immediately behind G is another lens to allow the image of the filament to be projected on the second photo-cell H.

Paired filters are placed in front of the cells to restrict the light to a very narrow wave-band.

The instrument may be used to compare the optical density of two solutions (as in a colorimeter) or it may be calibrated against a series of standard solutions and the optical density/concentration graph plotted.

In the first method of use, the darker solution is placed in cup G and the graduated aperture is set to zero. Distilled water is placed in cup B and the iris diaphragm so adjusted that there is no current flowing in the circuit (i.e. the light on the two cells is equal). Without altering the settings on the instrument, the lighter solution is now placed in cup G (or a similar
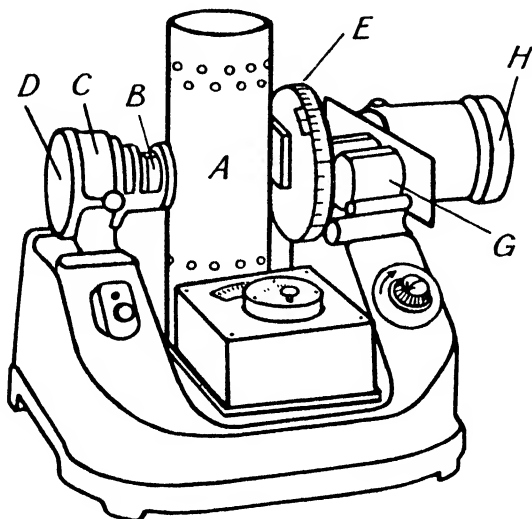
Fig. IV.9. Photo-electric Absorptiometer.

one mounted on a slide). The calibrated aperture is now reduced until again the galvanometer reading is zero. The reading on the wheel is taken and the respective absorption value of the two solutions is thus obtained.

In the second method a series of standard solutions is prepared and their respective optical densities are obtained as follows. A solution is placed in cup G, the graduated wedge is set at zero, and the iris on the other side of the system is adjusted (distilled water being placed in cup B) until a null-point is given. The solution in cup G is replaced by distilled water and wheel E is racked down until a null-point is again obtained. The graduation on the wheel will then be equivalent to the optical density of the solution. Readings are made for all the standard solutions and a reference graph plotted which relates optical density to concentration.

Precautions in the Use of Photo-electric Colorimeters. Photo-electric instruments are not "colorimeters" in the true sense of the word, since they do not measure colour or tone, but simply the amount of light absorption of a solution. For this reason, they are better called *"absorptiometers."* Without doubt, their introduction represents a great advance in colorimetry, but since they measure total absorption, there is the danger that variations of tint in solutions or the appearance of a precipitate might be overlooked. It is obvious that two solutions cannot be compared if one contains a precipitate which produces a turbidity as well as a colour. Yet the robot electric eye will measure all this as colour, and so tend to give an enhanced result. It is essential, therefore, when using these instruments to make a visual inspection of all specimens to ensure their complete freedom from turbidity when measurement of colour is desired.

On the other hand, photo-electric instruments can be used to measure turbidities (or rather light absorption due to turbidity). This widely increases their scope, for they may take the place of nephelometers (pp. 250–252 and 269–273).

In measuring a colour which is produced in an already tinted solution, the photo-electric instrument is very useful, since if two readings are made, one before and the other after the production of the second colour, then the different reading will be due solely to absorption by the latter.

Photo-electric cells are particularly sensitive to the infra-red region and for this reason should never be used without some form of infra-red filter. Of these, copper sulphate solution is most effective. With appropriate filters they can also be used for the measurement of absorption in the near ultra-violet.

NEPHELOMETRY

Nephelometry is the measurement of turbidity in liquids. Use is made of the fact that particles in suspension cause a scattering of a beam of light. An apparatus wherein the scattered light is projected into an eyepiece and a matching of intensity is made is called a "nephelometer."

In the same way that a colorimeter measures colours, so a nephelometer measures turbidities. It differs from the colorimeter, however, in that it compares scattered light and not transmitted light.

The nephelometric method may be used to assess almost any quantitative reaction where a highly dispersed and colourless precipitate is formed, *provided that the particle-size of the precipitate is constant and reproducible.*

Nephelometry was originally developed by Richards and Wells, who utilised the work of Tyndall and Rayleigh in elaborating the rationale.

The expression

$$T = K \frac{cld^3}{d^4 + \alpha\lambda^4} \dots\dots\dots\dots\dots\dots\dots\dots (1)$$

is given as relating turbidity (T) to concentration (*c*), wherein

> K is a constant depending on the medium;
>
> α is a constant for the method;
>
> *l* is the depth of liquid;
>
> *d* is the diameter of the suspended particles;
>
> λ is the wavelength of the light.

This equation is only valid for thin layers and dilute suspensions. It will be seen that the wavelength λ of the light and the diameter *d* of the particles both have a very marked effect on the result.

From this equation it follows that the turbidity is at a maximum when

$$\frac{d}{\lambda} = (3\alpha)\frac{l}{4} \dots\dots\dots\dots\dots\dots\dots\dots (2)$$

Consideration of equation (1) will show that Beer's Law will hold for these dilute suspensions, since when the turbidities of two *similar* suspensions are equal (*d*, α, and λ being constant), then

$$K \frac{cld^3}{d^4 + (\alpha\lambda)^4} = K \frac{c^1 l^1 d^3}{d^4 + \alpha\lambda^4} \dots\dots\dots\dots\dots (3)$$

$$\text{I.e. } \frac{c}{c^1} = \frac{l^1}{l} \dots\dots\dots\dots\dots\dots\dots (4)$$

That this is not usually obtainable may readily be demonstrated by experiment, and in application it is essential to utilise a correction, which in the main compensates for variation in particle size d.

Where the Duboscq or similar type nephelometer is used, this correction may be made by using for the constant K the expression

$$K = \frac{r(ry-x)}{x(r-l)} \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (5)$$

where $r=$ratio of dilute to concentrated standard

                           $y=$height of dilute standard

                           $x=$height of concentrated standard

If a series of suspensions are prepared and compared with the most concentrated at height x, then y and r will vary and the factor K may be computed.

A simpler method of overcoming these abnormalities is to prepare a wide series of suspensions, compare with the most concentrated, and plot the nephelometer reading against concentration. The graph produced may then be utilised as a reference, provided that the subsequent suspensions are prepared in a manner identical with that originally used.

Occasionally errors occur in nephelometry due to an effect first observed by Tyndall, who showed that scattered light from a very fine bluish suspension was polarised at right-angles to the illuminating beam, but that as the particles become larger, so polarisation is reduced. This wavelength effect, of course, upsets the mathematical consideration given above, but can be compensated for if the empirical method of constructing a calibration graph is utilised.

The Duboscq type of colorimeter (fig. IV.4) may readily be utilised as a nephelometer. Since the instrument is to measure scattered light, then the light path must be arranged so that the rays enter the side of the cups at right-angles to the plungers instead of through the bottoms. In this way light which is scattered at right-angles by the particles in suspension is projected into the eyepiece. It is, of course, necessary that the plungers be shielded so that only light projected upwards through their transparent ends is measured in the eyepiece. For the same reasons the bottoms of the cups are made opaque.

To use the instrument, a standard suspension is prepared and placed in one cup. The unknown solution is treated in an identical manner and placed in the other cup. The plunger in the standard suspension is lowered to a definite setting and the plunger in the other is then raised or lowered until an exact match of light intensity is obtained. The scale reading is then read off a previously calibrated graph or subjected to the instrument correction mentioned above.

There are a number of nephelometers other than the Duboscq type. Perhaps the most accurate is that of Kleinmann, in which the area of light

falling upon each tube is varied by means of a calibrated aperture. In this way the variability in depth of plungers is eliminated, and an accuracy of 0·5%, with linear relationship, is claimed.

Uses of Nephelometry. Nephelometric methods are of great value in reactions where no specific colour is obtainable, but insoluble precipitates form. Richards and Wells worked extensively on the estimation of silver chloride by the turbidimetric method. It has been used for both silver* and chloride assessment.† Barium sulphate precipitates are particularly suitable for nephelometric measurement, and in consequence these techniques may be used for the estimation of *barium* and of *sulphates*. Alkaloid *phospho-molybdates* are very insoluble, and by nephelometric procedures as little as 1 part in 300 million may be estimated.

There are, however, many difficulties with nephelometric technique, the chief of these being inability to produce a reproducible suspension of some stability. For this reason nephelometric methods have gained very little favour with analysts, and in consequence their applications are somewhat limited. These difficulties and the means of combating them are discussed in detail in the section on elaboration of the experimental method (pp. 269–273), and it is certain that if due care be made to the points therein enumerated, nephelometric procedures would have a much greater vogue.

Besides using the orthodox type of nephelometer for comparing two suspensions, the absolute densities of suspensions may also be measured by means of photometers. This is in fact *turbidimetry*, or measurement of the light transmitted through a suspension. Instruments such as the Pulfrich step photometer, the Vernes-Bricq-Yvon photometer, and any type of photo-electric colorimeter, wherein extinction of the light by the suspension is referred to a standard wedge, calibrated aperture, or a galvanometer reading, may be used for this purpose. It is obvious that in order to compare two suspensions their colours must be similar, although even this is not absolutely essential if screens are incorporated into the instrument so that the effect of colour is neutralised.

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* Richards, T. W., and Wells, R. C., *Amer. Chem. J.*, 1904, **31**, 235.
† Obermer, E., and Milton, R., *Biochem. Zeit.*, 1932, **251**, 329.

FLUORIMETRY

Theoretical Principles*

Many substances are capable of absorbing radiant energy and of re-emitting, in all directions, fluorescent radiation, which has wavelengths characteristic of the light-absorbing molecules and not only that of the incident beam.

Fluorescence is exhibited by many gases, solids, pure liquids, and solutions, and can be observed most clearly by inspection of a material in a direction at right-angles to a parallel beam of incident light. Hence the techniques of fluorimetry and of nephelometry are very similar in practice, though quite different in theory. Nephelometry depends upon the scattering of light, without change of wavelength, by colloidal suspensions, and makes use of a physical phenomenon largely independent of the chemical nature of the dispersed particles. Fluorimetry depends upon the physico-chemical properties of particular molecules, and can be used to detect and to measure quantitatively specific solutes in filtered homogeneous solutions. Its use is therefore restricted to the examination of a limited range of substances, such as the salts of uranium or of some "rare-earth" elements, complex aromatic or heterocyclic dyestuffs, amongst which, however, are several important drugs (e.g. acriflavine, mepacrine), biological products (e.g. oxidation products of certain vitamins of the B group), and indicators of wide applicability in analytical chemistry.

Since as a general (though not an invariable) rule the radiation emitted by a fluorescent substance has wavelengths equal to or longer than that of the incident radiation, many substances become markedly fluorescent only when exposed to ultra-violet light.

The exact relationship between the intensity and the wavelength of the incident radiation and the intensity of emitted fluorescence is not mathematically simple even in the case of a pure gas, and it is still more complex for solutions, the chief factors which have to be taken into consideration being: (a) the degree of absorption of the incident radiation; (b) the "quenching" of the fluorescence of the activated molecules by collision with each other or with solvent or solute molecules; and (c) the simultaneous occurrence of photo-chemical decomposition. Nevertheless, when a gas or a very dilute solution is being examined under conditions in which only a small fraction of the incident radiation is absorbed, the intensity of re-emitted fluorescence is, over a wide range of concentrations, directly proportional to the concentration of the fluorescent substance. As with nephelometry, therefore, comparison methods can be used quite accurately to analyse micro-chemical quantities of fluorescent substances, though

* See Bowen, E. J., "The Chemical Aspects of Light " (Oxford, 1946).

absolute measurements of the intensity of fluorescent radiation are rarely of value. Other coloured substances or suspended particles, which may absorb or scatter the fluorescent radiation before it can be observed, should, of course, be eliminated. On the other hand, observation through selective colour-filters often enhances both the sensitivity and the accuracy of analysis.

Uses of Fluorimetry

1. Substances which fluoresce in visible or in ultra-violet light can be estimated directly. Certain gases at low pressure, as for example mercury vapour, emit a characteristic fluorescence, termed a "resonance vibration," when illuminated by light of a particular wavelength, and can be estimated by this means; whilst many colourless solid or dissolved substances become brilliantly fluorescent when illuminated by ultra-violet light. In seeking for a method of analysis of a complex organic product it is always worth while to inspect its dilute solution, in an aliphatic solvent such as alcohol or acetic acid, under the light of an ultra-violet lamp.

2. Fluorescent substances can often be produced by simple chemical reactions which can be developed into quantitative analytical procedures. Thus oxidation with alkaline potassium ferricyanide of solutions containing aneurin (Vitamin B_1) produces a product, thiochrome, which has a characteristic blue fluorescence. Again, saccharin will condense with phenolic substances, such as resorcinol, to yield fluorescent sulphon-phthalein dyes.

3. Fluorescent indicators can be used in many ways. In Part III they have been referred to in connection with acid-alkali titrations and precipitation reactions. These titrimetric methods can be made much more sensitive by using accurate comparators. Still more minute amounts of many substances can often be estimated by taking account of the formation of fluorescent lakes, or by precipitating coloured or fluorescent reagents from solution.

4. The fluorescence of a substance can often be used as an index of its purity or as a guide to the efficacy of its separation from contaminants. Adsorption, and chromatographic, techniques (pp. 260–261) for the purification of minute amounts of material can often be effected quite delicately with colourless substances with the aid of an ultra-violet lamp. Individual components of hydrocarbon oils can be examined in this way by fractional adsorption on activated alumina.

Errors of Fluorimetry

1. Fluorescent solutions cannot be compared accurately by direct observation in a glass tube, since an unknown, and variable, amount of the unchanged incident light also enters the eye. Comparison is usually made in a shielded instrument in which the only incident light falls upon the solution in a direction accurately at right-angles to the direction of observation. Good

results can be obtained by observation down a long tube provided with stops, though for precision work the incident light should pass through a collimator. If provided with suitable screens, however, the Spekker photo-electric absorptiometer (p. 248, compare p. 275) can be used as a fluorimeter.

2. The fluorescent solution should not be deeply coloured; otherwise a significant proportion of the fluorescent radiation will be absorbed before it can be measured. If the fluorescence of coloured solutions has to be examined, then the two solutions should first be balanced in a dilution colorimeter. Colour-filters can also be used to overcome this defect.

3. Turbid solutions should never be examined. Otherwise scattered light will interfere with the true fluorescent radiation.

4. Many substances, even if present in comparatively small amounts, have the power of "quenching" the fluorescence of solute molecules. It is inadvisable, therefore, to estimate a dissolved substance by means of its fluorescence without first purifying it, as far as is possible, from contaminants. Iodides, phenolic substances, aromatic amines, and some proteins frequently have this "quenching effect." Many errors can be ascribed to the adsorption of the required product upon the surface of a colloidal protein.

5. Photo-chemical decomposition may introduce serious errors if substances are subjected to prolonged exposure to ultra-violet light.

6. Sometimes colourless substances may produce errors by absorbing ultra-violet light, and so hindering the emission of fluorescent radiation.

CONSIDERATIONS AND PROCEDURE USED IN THE DEVELOPMENT OF ACCURATE COLORIMETRIC MICRO-METHODS

SINCE colorimetric methods will doubtless be used to an increasing degree in future, it is essential that every analyst should acquire a very clear idea of the procedures necessarily to be adopted in developing from a colour reaction an accurate analytical process.

CRITERIA FOR A SATISFACTORY COLORIMETRIC ANALYSIS

A colour reaction should fulfil the following requirements before it be adapted for analytical use.

1. Specificity of the Colour Reaction

For satisfactory application in analytical chemistry, the colour that is produced when a solution to be analysed is treated with a colour-producing reagent must be due only to the substance which is to be estimated. No similar colour should be given by any other substances which might also be present in the solution.

Thus dimethyl glyoxime gives with nickel a red co-ordination compound which may be used for colorimetric analysis, but other metals, e.g. copper, also give coloured products with this reagent. It is clear, therefore, that dimethyl glyoxime cannot be used for the colorimetric estimation of nickel in the presence of copper.

In point of fact, very few reagents are specific for a particular substance, but a large number are relatively specific in that they give colours with a small group of chemically related substances only. By utilising certain devices, the analytical process may often be made specific for a single substance. Applications of dithizone (see pp. 284–286) exemplify this. Dithizone forms coloured co-ordination compounds with a large number of metals, but (a) by the use of another complex-forming substance, such as cyanide or thiocyanate anions, and (b) by careful adjustment of the pH it can be used as a quite specific reagent for an individual metal.

2. Proportionality between Colour and Concentration

Beer's Law holds for most coloured solutions: certain colloidal dispersions and coloured lakes are exceptions (see **6** below). But it does not necessarily follow that the depth of a colour is directly proportional to the molar concentration of the substance to be estimated. This is an analytical factor

and not an optical one, for the relationship between colour and concentration depends upon the chemical process concerned.

For instance, colour formation may be due to a reversible chemical reaction, e.g. $FeCl_3 + 3NH_4CNS \rightleftharpoons Fe(CNS)_3 + 3NH_4Cl$, and may thus be a complex function of the concentrations of both the substance to be analysed (Fe) and of the reagents used (NH_4CNS).

If visual colorimeters are to be used, then it is essential that the colour intensity should increase linearly with the concentration of the substance to be estimated over the concentration range used in the analysis. This pre-requisite is not so necessary if photometers or photo-electric instruments are used, since a calibration curve may be constructed to relate the instrument reading of the colour with the concentration of the substance.

3. Stability of the Colour

It is essential that the colour produced in an analytical reaction should be sufficiently stable to allow an accurate reading to be taken. Some colours are very transient and tend rapidly to reach maximum development and then to fade. With such reactions, the reading should be taken at the peak of colour development, and it follows that if the time interval over which this occurs is short, then difficulty will be experienced in obtaining reliable, reproducible data.

Since rates of colour development are usually functions of the temperature, and of the concentrations of the reagents, these factors must be rigidly controlled whenever a transient colour is involved.

It is occasionally possible to slow down the reactions responsible for colour fading, or even to terminate colour development at a particular point, by adding, after a fixed time, further reagents which destroy or neutralise one of the reactants.

4. Effects of Other Substances on the Colour

A full knowledge is necessary of the effects of foreign substances in the solution upon the colour reaction. Hence a preliminary qualitative analysis may be advisable. Besides imparting a colour to the solution or actually producing a colour with the reagent used, a foreign substance may interfere with the reaction between the substance to be analysed and the colour-producing reagent. An example of this is to be found in the estimation of iron as thiocyanate: fluorides, if present, produce complexes with the iron and so prevent its reaction with the thiocyanate. Again, there may be present a substance which oxidises or in some other way destroys the colour-producing reagent. It is particularly important to guard against this error when using any of the special organic reagents for the colorimetric analysis of metals.

Foreign substances may also modify the reaction rate by their mass action effect, or by buffering the solution to an abnormal pH may cause modified

colour development.   Other possibilities include the formation of lakes and the absorption of the colour by certain colloids.

If a colorimetric method is to be made accurate, cognisance must be taken of facts such as these and the process modified accordingly.

5. Reproducibility

An accurate colorimetric method must be capable of giving reproducible results, but it is not essential that every colour reaction should be a stoichiometrically quantitative chemical change.   In practice, the reproducibility of a colorimetric process can only be relied on when the analyst understands all the many factors which may modify the depth of colour and knows how to control them experimentally.   Besides the criteria mentioned in **3** and **4** above, there are the effects of temperature, concentration of reagents, time of colour development, rate of fading, stability of the colour in air and light, and certain others.

Reproducibility must not be an individual affair, for the method must be capable of giving good results in the hands of any skilled technician.

6. Clarity of the Solution

Before a colour may be accurately compared with a standard in a colorimeter, it is essential that the solution should be quite clear and free from any precipitate.   The effect of a turbidity is to scatter as well as to absorb light, and this renders impossible colour comparison with a clear standard. The result is immediately obvious when a solution is viewed through a colorimeter eyepiece, since the effect of a turbidity is usually to modify the colour tone.   When such a solution is measured in a photometer or in a photo-electric instrument, this point is apt to be overlooked, for a false reading corresponding to the net transmitted light will otherwise be obtained.

METHODS FOR OBTAINING A SPECIFIC COLOUR

It is essential when estimating a substance by a colorimetric process to make sure that the colour is specific for the substance to be analysed.   Most colour reagents, though selective, are not specific, but may be rendered so by correctly controlling the operational procedure.   The methods used to obtain specificity are in the main twofold.   First, preliminary steps may be taken to effect the isolation of the substance under review; secondly, the reaction conditions can be adjusted to bring about the suppression of the colours given by all other substances than the one to be estimated.   The particular method that should be used varies with the substance to be analysed, and illustrations of these are given freely in Feigl's monograph on "Spot Tests."

Methods of fairly general applicability are discussed below.

A. Separation Prior to Colorimetric Analysis

The usual method of separation of metals, as used in group analysis, often fails when applied to minute quantities of substances. This may be due to solubility of the precipitate when present in small amounts, supersaturation of the solution with the substance, or, very frequently, to the formation of a non-coagulable colloidal precipitate, or to its peptisation. In these instances inducement to precipitate can be made in one of the following ways:

1. Precipitation with a "Collector"

A "collector" is a substance added to a solution which, on precipitation with a reagent, takes down with it traces of a product which would otherwise remain in suspension or in solution. "Collectors" act by entraining the substance by adsorption, by initiating precipitation from supersaturated solution, or by mixed crystal formation.

TABLE IV.2

COLLECTORS USED IN MICRO-ANALYSIS

Collection by Co-precipitation

Precipitation of PbS		may be used to collect traces of		CuS
,,	Ag_2S	,,	,,	PbS
,,	CuS	,,	,,	PbS, ZnS, MoS_2
,,	As_2S_3	,,	,,	HgS
,,	Te	,,	,,	Au (as AuTe).

Collection by Adsorption

Fe-(Cupferron)	may be used to collect traces of		Ti, V, or Zr
Al-(8-Hydroxyquinoline)	,,	,,	Co
$SrSO_4$ or $BaSO_4$	,,	,,	Pb or Ra.

Collection by Mixed Crystal Formation

Deposition of $MgNH_4PO_4$ may be used to collect traces of As.

2. Separation by Adsorption

(a) *Adsorption by slightly soluble substances.* One specialised way of "collecting" micro-chemical quantities of a substance to be estimated in a large bulk of solution consists of adding to the solution a slightly soluble compound capable of reacting with the substance to be separated, so that the minute precipitate of the required substance is adsorbed on to the excess of the insoluble reagent.

For instance, if solutions containing small amounts of the noble metals, and other readily reducible substances such as tellurium, selenium or arsenic, are shaken with solid mercurous chloride, then reduction to the elementary state occurs and the product is adsorbed on to the excess of mercurous chloride.

In a similar manner, papers impregnated with cadmium sulphide can be

used to remove traces of copper from solution, and in neutral solution lead may be removed by passage through a paper containing zinc sulphide.

Lead can also be separated by adsorption of its carbonate on calcium carbonate, or of its sulphate on calcium sulphate. In the latter case alcohol should be added to suppress the solubility of the calcium sulphate, and the adsorbed lead may subsequently be removed from the sulphate precipitate by extraction with ammonium acetate.

In this type of separation, which depends upon surface adsorption after precipitation, the most effective adsorbing surface is made available if the collector is precipitated *in situ* instead of being added to the solution in solid form.

(b) *Adsorption followed by complete elution.* Organic substances may frequently be separated from dilute solution by shaking the latter with a reactive solid, such as silica gel, kaolin, or charcoal. Subsequently the required substance can be recovered by eluting the filtered adsorbent with a liquid of different pH, by a different solvent, or by a reagent in which the desired product dissolves. Thus adrenalin in biological fluids can be separated from other phenolic substances by adsorption on to silica gel and elution with weak alkali.

The chromatographic technique of filtration through a column of adsorbent is a very convenient one, even though it may be impossible to watch the process visually.

Synge's method of amino-acid separation* illustrates the great value of this technique for the quantitative fractionation of complex mixtures.

3. Chromatographic Separation†

As in other fields of chemistry, chromatography is an invaluable technique for separating mixtures prior to colorimetric analysis. Although most useful for the separation of organic substances (e.g. the carotenoid pigments), it may also be employed for inorganic analysis. Thus traces of iron may be separated from copper or traces of copper separated from cobalt by chromatographic technique.

The method consists of passing the liquid under examination through a long tube packed with the adsorbent. Preferential adsorption of particular substances will occur at different layers in the column, and if a colourless adsorbent, such as activated alumina, silica gel, or kaolin is used, then the whole process can easily be controlled. Subsequent to the initial extraction it is customary to "develop" chromatogram by passing pure solvent through the tube, when, owing to re-solution and subsequent re-adsorption from more dilute solution, distinct zones of differently adsorbed substances are

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* Synge, R. L. M., *Biochem. J.*, 1939, **33**, 1913-31; 1941, **35**, 91, 295, 1358; 1943, **37**, 79, 86, 313.
† Zechmeister, L., and Cholnoky, L., "Principles and Practice of Chromatography" (trans. by Bacharach, A. L., and Robinson, F. A., London, 1941).

produced. Even when these zones are not clearly defined as coloured bands in the tube, they may often be distinguished by means of their fluorescence in ultra-violet light.

The following techniques may be used for separating the required product:

(i) *Separation by fractional elution:* the required substance may be dissolved out completely by passing through the tube a new solvent in which it dissolves easily. Solvent is passed through the tube until the whole of the required coloured band has been removed, and the total filtrate is then concentrated.

(ii) *Separation by fractional extraction:* after the adsorbed bands have been suitably developed by means of a solvent, the cylinder of adsorbent may be pushed out of the tube with a glass rod on to a sheet of clean glass or paper, and cut into sections with a razor blade. Each section may then be extracted separately in a Soxhlet.

(iii) *Partition Chromatography* is another rapidly developing technique which can be applied in many ways. For instance, a two-dimensional separation of a mixture can be effected on a paper sheet by successive elution in two directions at right-angles.* As yet this method has been used chiefly for the qualitative separation of analogous substances; e.g. mixtures of amino-acids resulting from protein hydrolysis or of the mono-saccharides obtained from a complex carbohydrate,† but its potentialities in quantitative micro-analysis are undoubtedly great.

4. Base-exchange Separation

Base-exchange substances are frequently used to separate small quantities of ions from solutions. This principle, which finds commercial development in water-softening, was developed from the discovery that certain insoluble zeolites (basic aluminium silicates) will exchange their cations with others in solution. Thus, small quantities of calcium can be removed from water by passing it through a pad of sodium permutite, when calcium ion is exchanged for sodium. The subsequent removal of calcium from the solid permutite may be effected by passing saturated sodium chloride solution through it. Iron may be removed from solution and eluted in the same way.

Another application of the use of permutite is in the estimation of amino-acids in biological fluids which contain ammonium salts. Passage of the fluid over permutite removes ammonium ions completely, and allows the selective colorimetric reagent sodium-β-naphthoquinone sulphonate to be applied in the specific estimation of the amino-acids in the filtrate.

A number of insoluble phenol-sulphonic acid/formaldehyde resins are now being developed for a similar purpose, and are sure to find analytical applicability. With certain of these synthetic products anion exchange is also possible.

* Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.
† Partridge, S., *Nature*, 1946, **158**, 270.

5. Electrolysis and Electro-dialysis

These procedures, which are described in Parts I and V (pp. 38–40 and 409), can often be used for the preliminary separation of traces of metallic substances. One useful micro-analytical application has been in determining traces of mercury in air, urine, and stools in cases of industrial mercurialism.* Mercury sulphide is carried down with a collector, e.g. copper sulphide, the precipitate is redissolved in aqua regia, submitted to electrolysis, using platinum electrodes, and finally the mercury is volatilised off, redissolved, and determined colorimetrically.

6. Separation by Selective Solvent Extraction

Although this process is widely used for the separation of organic compounds, it is not always realised how extensively it can be applied in inorganic analysis also. Thus ferric thiocyanate may be extracted from water with amyl alcohol, whilst ferric and auric chlorides can both be extracted with ether from 20% hydrochloric acid. Organo-metallic complexes can very often be separated by selective extraction. Thus dithizone (see pp. 45–47) forms with many metals compounds which may be extracted from water with carbon tetrachloride or xylene. Dimethyl-glyoxime, 8-hydroxy-quinoline, potassium xanthate, and α-nitroso-β-naphthol also give metallic co-ordination compounds which are soluble in organic solvents.

The blue reduction product of phosphomolybdate has been removed from solution by organic solvents prior to colorimetric estimation.

7. Separation as Gas

Occasionally it is convenient to separate a substance as a gas or by distillation. An obvious example of this is the colorimetric estimation of ammonia, where the gas is liberated, absorbed in acid, and then estimated by the Nessler technique. Again, in the estimation of potassium by the cobaltinitrite method (p. 305, compare p. 169) a preliminary treatment with nitrous acid is invaluable for effecting the removal of ammonia. Other examples include the separation of arsenic as arsine prior to colorimetric estimation (p. 278) and the sublimation of mercury, selenium, and tellurium from precipitates containing these substances.

Separation by distillation makes use of the same principle. Examples of this are: (a) separation of fluoride by distillation with perchloric acid (p. 191); (b) removal of silica as silicon fluoride; (c) distillation of arsenic as the trichloride, or of antimony as the pentachloride, in the presence of concentrated hydrochloric acid; (d) separation of chromium by distillation of chromyl chloride; and (e) distillation of selenium, and of tin, as volatile chlorides from solutions in strong sulphuric acid.

* Cf. Stock, A., *Ber.*, 1938, **71**, 550.

B. Methods for Making Colour Reactions Specific

1. Adjustment of pH

Often a reagent which reacts with a group of substances may be made specific for one of them by causing the reaction to take place within well-defined pH limits. One valuable instance of this has been discussed in connection with dithizone (pp. 45–47. Compare the examples given on pp. 284–286).

2. Selective Extraction

Sometimes a reagent which forms coloured complexes with certain compounds has a greater affinity for one than for the others. In this case it is often possible to extract an aqueous solution with successive small quantities of a dilute solution of the colour-producing agent in an immiscible organic solvent until the required component has reacted completely. Then, by subsequent addition of excess of the reagent to the residual aqueous layer, the other substances can be allowed to react separately. In this way mercury may be extracted from copper as the dithizone complex.

3. Formation of Complexes

The interference of foreign substances with a colour reaction may occasionally be overcome by adding to the solution to be analysed a substance with which the interfering body forms a non-reactive complex. Many instances of this are given in Feigl's monograph on "Spot Tests." Again, in analyses by means of dithizone (pp. 45–47) of the many metals which form coloured products, only lead, tin, and bismuth will react in acid solution in the presence of cyanide, since the other metals then form complex cyanides with which reaction does not occur.

Another example is afforded by the estimation of silica as silico-molybdate (p. 313). Arsenic and phosphorus will interfere, but if citrate be added to form complexes with these two substances, the method becomes specific for silica.

4. Oxidation or Reduction of Interfering Substances

Another device to obtain a specific colour from a selective group of reactants is to oxidise or reduce the interfering substances to non-reacting bodies. Iron, nickel, and cobalt are metals particularly amenable to this treatment.

5. Destruction of Interfering Substances

Sometimes interfering substances may be destroyed in solution prior to a colour reaction being attempted. Thus, in the colorimetric estimation of lactic acid (p. 324), the substance is first converted to acetaldehyde, which is then made to give a colour by reaction with veratrol. Sugars also give

this colour reaction, but if a pre-treatment with copper sulphate and lime be carried out, then the sugars are destroyed and so do not interfere.

6. Employment of Colour Filters and Methods of Compensation

Where the colour of a solution is due to more than one substance, then the difficulty may sometimes be overcome by making the colorimetric assessment with light of a well-defined wave-band, so chosen that the interfering substances do not absorb light of the tint selected. When simple forms of colorimeter are used, filters in the eyepiece will ensure this. Again, quite accurate results can be obtained by adding to the comparison standard the interfering coloured product in equal quantity to that present in the unknown solution. Alternatively, if the solution is coloured prior to the addition of the colour-producing reagent, then by taking readings both before and after addition of the reagent, assessment by difference may be made.

Sometimes it is possible to produce the colour required, and then selectively to destroy this, or the interfering colour, when again a significant result may be obtained by making two colorimeter readings. Any of the methods classified under Sections 2 to 5 above may be used for this purpose.

THE ATTAINMENT OF REPRODUCIBILITY

Every colorimetric method should pass the test of giving reproducible results before it can be accepted as accurate. Moreover, it should be ensured that different analysts can get concordant results when each follows the specified instructions. In elaboration of techniques, therefore, all the factors which might lead to variations in colour tint or intensity must be strictly controlled.

After having chosen a colour reaction which is selective, and by one or other of the various processes enumerated above having made it specific for the substance to be estimated, the next step is to find the effect of possible variables, such as the following, on the colour reaction.

1. Concentration of Reagents

The mass action law applies to all colour reactions, but when micro quantities are involved it is usually sufficient to make certain that there is a many-fold excess of the colour-producing reagent.

Frequently the concentrations of reagents employed have a marked effect not only upon the depth of colour but also on the specific nature of the reaction. An instance of this is found in the estimation of phosphates by reduction of phospho-molybdic acid with stannous chloride (p. 314). It is essential in this method that the concentration of the sulphuric acid in the final solution should be close to $1 \cdot 0N$, and that in any case its deviation

from $1 \cdot 0N$ should be identical in the standard and in the unknown. Increased acidity reduces colour intensity; decreased acidity produces an increase in colour. Similarly, variation in the concentration of molybdic acid affects the result. Finally, it is vital that the strength of the stannous chloride solution should be maintained within fixed limits, since auto-reduction of the molybdic acid itself will occur with high concentrations of stannous chloride, whilst incomplete reduction of the phospho-molybdic complex occurs when the stannous chloride concentration is low.

The above example is by no means an extreme case of a complicated reaction which can, however, be made into a trustworthy micro-analytical procedure.

Precautions should therefore be taken when elaborating a colorimetric method to ascertain the effect on the colour of variations in the amounts of all the reagents involved, not only for one concentration of the substance to be estimated but over the whole range to be encountered.

2. Development Time

Some colour reactions occur quantitatively immediately upon the addition of the appropriate reagent, but it is frequent to find that a definite time interval occurs before the colour reaches its maximum. It is essential that a study should be made of the rate of development of colour when such a method is being elaborated. This is most easily carried out by using objective, or photo-electric, instruments, since it merely involves correlating the extinction coefficient with time, by taking readings at, say, minute intervals. If a visual colorimeter is used, then it is often advantageous to make time interval readings against a permanent colour standard, such as a tinted glass plate (p. 227).

Having once fixed the time for production of maximum colour, it is best to arrange that this period is subsequently allowed when making any analytical readings.

Occasionally colour reactions reach the optimum point of colour development and then the colour begins to fade. It is therefore necessary to measure fading rate as well as development rate.

In all cases where there is a noticeable delay in the production of the colour maximum or some tendency to fade, the comparative colorimeters offer definite advantages over the objective instruments, since the development, or fading of the colours of both the unknown and the standard solutions are likely to be parallel: if objective instruments are used, then the calibration graph will refer to the time-development conditions used when preparing that graph only, and unless all subsequent estimations are made strictly on the basis of that same time interval, then inaccuracies will result.

If the colour is due to a colloidal solution, then it occasionally happens that the colloid ages and precipitates after a period. This must be avoided

at all costs, since if there is any trace of precipitation, colour comparison is quite impossible. In this contingency two procedures are available. First, colour comparison can be made before any precipitation occurs, or secondly, means may be employed to prevent precipitation, e.g. by addition of a protective colloid or other peptising agent. This latter method is frequently used in colorimetric technique (see pp. 233, 281–284) and often allows of the estimation of a clear coloured solution where otherwise only a precipitate would result.

Sometimes colour development, besides being proportional to the concentration of the substance to be estimated, is a direct function of the reaction time. This situation may be dealt with by taking the colour reading at a fixed time after mixing the reactants, but it is sometimes practicable to block the development of colour by adding to the reacting mixture, after a specified time, a further reagent which destroys or inhibits one of the essential reactants. An example of this is to be found in the estimation of urea in urine (p. 345), wherein urea is used to catalyse the reaction between furfural and stannous chloride in strong acid solution. The reaction here is a slow, continuous process which, after a given time period, can be stopped completely by addition of a sodium acetate buffer.

Development time is often influenced by variations in the concentrations of reagents, and the procedures considered in the previous section should all be regarded in this light.

3. Temperature Control

Colour reaction may be greatly influenced by temperature. Some reactions proceed so slowly at room temperature that it is essential to heat the reaction mixture to produce any measurable colour within a reasonable time interval. An example of this is shown by the reduction of phosphomolybdic acid with bisulphite, where the same depth of colour is produced by boiling for 5 minutes as is obtained on standing at room temperature for 24 hours. Under circumstances such as these, the optimum heating temperature as well as the time of heating must be investigated.

4. Effects of Other Substances

In the elaboration of a colorimetric method, effects upon the colour reaction of substances other than the one to be estimated must be studied thoroughly. Likely variations may be due to:

(a) *pH Changes*. Most colour changes are sensitive to pH changes in the solution if these are very gross, but some reactions are affected by quite small variations of pH. In these cases it is necessary to add an appropriate buffer.

(b) *Organic Substances in Solution*. Sometimes organic substances, e.g. proteins, gums, tannins, etc., can markedly affect a colour reaction and even

change the tint by adsorption effects. In the vast majority of the colour reactions of metallic ions, the presence of substances such as these is definitely precluded, but even in cases where it is not immediately apparent that organic substances interfere, care must be taken, nevertheless, to verify that their effect is negligible. With certain dyestuffs, lakes may be formed if heavy metals are present, and the intensity of the lake may vary with an increase in the concentration of either of the components.

(c) *Presence of Electrolytes.* "Salt effects," due to high concentrations of other electrolytes, often influence colour reactions considerably, and variations in the concentrations of other salts commonly encountered in solutions to be analysed should always be investigated. Sometimes the "salt effect" can cause the precipitation of a colour which normally would remain in true solution.

(d) *Presence of Substances of Similar Nature.* Although a reaction may be considered as specific for a given substance, it often happens that analogues have a noticeable effect if present in high concentration. It is therefore essential to ascertain the effects, on any specific colour reaction, which may be due to large amounts of substances similar to the one being analysed, and to determine the limits, if any, of freedom from interference.

(e) *Complex Formation.* Certain colour reactions may be inhibited completely by the presence of complex-forming substances. Citrates, tartrates, fluorides, and similar anions can have particularly marked effects upon colour reactions for metallic ions. It should generally be regarded as an essential pre-requisite that a solution should be freed from these interfering substances before the colour reaction is carried out, but the effects which can occur and the concentration limits possible before significant modification of the colour intensity occurs should always be noted. It is, for instance, impracticable to carry out a fluoride separation before every analysis for a heavy metal, though it may often be advisable to test for the absence of a significant concentration of fluorides before conducting quantitative micro-analysis for "trace elements."

EXPERIMENTAL PRECAUTIONS IN COLORIMETRIC ANALYSIS

In carrying out colorimetric analysis, great care should be taken to avoid *contamination* at all stages. As with micro-techniques generally, a small contamination may lead to a serious error.

1. Scrupulous care should be taken with the cleaning of glassware. Traces of materials adsorbed on colorimetric cells can cause very great errors.

2. The common practice of placing the thumb over the top of a tube before shaking to mix the contents is definitely to be deprecated. For instance, in the estimation of blood sugar (see pp. 210, 334) errors of up to 100% may be introduced in this manner.

3. Metals may easily be introduced into the solution from laboratory equipment: thus iron may come from retort stands, copper and zinc from Bunsens, aluminium from clamps, whilst mercury, antimony, zinc, and sulphur can all be extracted from rubber tubing. Agate mortars are preferable to steel or porcelain ones for crushing solids, and metal sieves should be avoided.

4. Silica apparatus is often desirable, since it is very resistant to most acids and does not give off traces of many elements, as is the case with glass and porcelain. Fluorides are often an unwelcome contaminant, since fluxes of this substance are used with most glasses and for graduation purposes hydrofluoric acid is invariably used. This element may also be introduced from thermometers, and it is removed only with great difficulty. Hence very careful blanks must be taken when examining for fluoride.

5. Filter-papers are a troublesome source of contamination in trace-metal analysis, and blanks should be made, particularly if the paper is to be ashed with a precipitate.

6. Mercury is an invariable contaminant in all laboratories. Up to 100 μg. per cubic metre is a very common concentration in laboratory atmosphere, and the greatest precautions must therefore be followed when testing for this element.

7. Copper may be introduced with distilled water, and when analysis is to be made for this element, redistillation from glass apparatus is essential. When extreme sensitivity is called for, distilled water should be stored in silica vessels.

On the other hand, *losses* during the preliminary analytical procedure must be guarded against. Thus:

1. On ashing a sample there is often left a siliceous residue which is most retentive of traces of metallic elements, particularly iron and aluminium, which may be recovered completely only after removal of the silica by treatment with hydrofluoric acid.

2. Filter-papers may adsorb a considerable proportion of the substance to be estimated and removal may involve alternate acid-alkali extraction.

3. It is a common experience when ashing to lose some of the substance by fixation into the glaze of the crucible. For this reason only unscratched dishes, preferably of silica, are to be recommended.

4. In dilute solution some metals are strongly adsorbed on to glass. Mercury affords an example of this, since dilute solutions of mercury salts (containing up to 10 μg. per millilitre) may be cleared completely after storage in glass for 24 hours. Prolonged storage of very dilute solutions is always to be deprecated. Standard solutions for comparison purposes should always be prepared, when required, by diluting stock solutions of not less than $M/50$ concentration.

ELABORATION OF NEPHELOMETRIC
METHODS

NEPHELOMETRIC or turbidimetric methods would have much more general application were it not for the difficulty of preparing reproducible suspensions or dispersions.

Although in general it is easier to obtain specificity with precipitation reactions than with colour formation, many difficulties are encountered in controlling the physical nature of suspensions.

Whilst in theory almost any reaction which produces an insoluble precipitate is capable of being adapted to nephelometric purposes, in practice it is, most frequently, the amorphous precipitates which are suitable for comparative measurement. Substances which precipitate in crystalline form do not usually yield reproducible dispersions, because while the light scatter, or transmittance, is primarily proportional to the concentration of the precipitate, it is also affected by the particle size. With crystalline substances the mean particle size is affected so much by slight variations in the conditions of crystal formation that reproducibility of optical properties is almost impossible.

Many amorphous precipitates cannot be subjected to nephelometric measurement because of the great difficulty of keeping a suspension evenly dispersed for long enough to obtain an accurate reading. Precipitation is usually a gradual affair, commencing with the appearance of very small particles which gradually grow individually and also tend to coalesce or "clump" into a smaller number of larger aggregates. The optical density of any dispersion therefore changes with time until the separation of solid is complete and the coagulation rate has become very low.

Since the rate of sedimentation of each particle increases with its size according to Stokes' Law, by the time that a precipitation reaction is complete much of the material may have deposited on the bottom of the receptacle. Shaking the product to resuspend a precipitate and to break up aggregates can be helpful, but must of necessity be a factor for investigation in every case.

Much of the difficulty of obtaining a reproducible suspension may be overcome by the incorporation of a *protective colloid*. This usually consists of a gum, a protein, or a similar macro-molecular substance, which, by surface adsorption, has the effect of preventing the small particles of a precipitate from coagulating into larger aggregates. Thereby the mean particle size is kept low, and this increases greatly the time of stability of the dispersion. Gum arabic is often employed for this purpose, usually in about 5% concentration. Gum ghatti (first used by Folin for use with

Nessler reagent) is usually much more suitable and has a protective power of at least 200 times that of gum arabic. Gelatine is also used, and usually a 1% solution is quite effective in maintaining a precipitate in stable suspension.

Precipitation is invariably affected so much by the experimental conditions employed that in the development of any new nephelometric method many factors must be studied and a rigid technique must be chosen. Systematic consideration should always be given to the following:

1. Development of Technique and Range of Method

Having decided upon the reagent required to produce a particular suspension, it is next necessary to ascertain the limits of concentration to be studied. Nephelometry is only applicable to *very dilute* suspensions and instrument sensitivity becomes very small when the suspensions are above a certain optical density. Thus it is far more easy to compare suspensions having a light transmission of $(50\pm2)\%$ than suspensions having a light transmission of only 1–2%.

2. Specificity

The considerations affecting the specificity of the chemical reaction involved which have been discussed in detail under colorimetric methods (pp. 258–264) apply equally with suspensions, and in the main similar methods are employed to ensure that the optical density of the suspension is directly proportional to the concentration of the substance to be measured.

3. Protective Colloid

Where suspensions are readily coagulable, the use of a protective colloid should be investigated, for it is rare to find that this addition does not assist appreciably in the stabilisation of the dispersed phase. It is essential to study not only the effects of various available colloids, but also the correct concentration of the selected one that should be added to produce the optimum effect.

4. Effect of Electrolytes

The formation of a precipitate invariably follows the stages: (*a*) separation of colloidal dispersion, (*b*) coagulation of the colloid to give fine crystalline particles, (*c*) aggregation of small particles to larger agglomerates. All these processes are usually accelerated by the presence of electrolytes in the solution. For this reason it is essential that the effects of a number of the common ions on the precipitation process should be examined. When an effect is very marked, it is often a good practice to add so much electrolyte as to obtain the maximum salt effect and thus swamp any difficulties of reproduction caused by small variations in the salt contents of the samples to be analysed.

5. Manner of Addition of Reagent

The manner in which the precipitating reagent is added to the solution can markedly affect the type of precipitate formed and also its optical density. Generally speaking, the slow addition of a reagent (e.g. drop by drop, with shaking between additions, so that the substance to be precipitated is in excess until precipitation is complete) tends to produce a coarse granular precipitate which is unstable and rapidly settles. The addition of the reagent in a squirt (as from a syringe or pipette with bulb) tends to rapid admixture of the solution and to the production of a colloidal precipitate. To obtain reproducible results, therefore, it is essential that the suspension should be prepared by the addition of the reagents in a specific manner, and that the technique decided upon as most suitable should be utilised for preparing both the standard and the unknown dispersion when nephelometers are used, and also in the production of the standard graph when objective instruments are used. Since no suspension is permanently stable *a fresh set of comparison standards must always be prepared for each nephelometric analysis.*

6. Effect of *p*H

The *p*H of the solution often has a considerable effect upon the production and nature of any precipitate. With some reactions, of course, any deviations of *p*H will result in solubilisation of the precipitate, but even in reactions where the *p*H limits for precipitation are wide, small variations may markedly affect mean particle size and the relative optical density of the precipitate. If this is proven to be the case, then it is best to guard against such eventualities by the addition of an appropriate buffer to the solution.

7. Effect of Temperature

Just as the presence of electrolytes may affect the nature and stability of precipitates, so variations in temperatures of solutions may also cause differences. A precipitate which is fine and semi-colloidal at room temperature may easily be coarse and unstable if produced at, say, 35° C. Hence the preparations both of comparison standards and of unknown dispersions should be made at identical temperatures.

Sometimes heat is necessary to induce precipitation. An instance of this is in the production of silver chloride in the presence of a protective colloid (see p. 396). In cases such as these it is necessary to study the optimum heating conditions to obtain reproducible results and to ensure that these actual conditions are used when the method is employed in analysis.

Similarly, increase in temperature may cause solubilisation of a precipitate: in such cases it is often helpful to specify that the precipitation is carried out at a certain fixed temperature.

8. Development Time

Precipitation is rarely immediate. Usually the successive stages of precipitation, mentioned above (p. 270), occur during a time interval which varies not only with the nature of the substance but with such other variables as temperature, presence of electrolytes, manner of addition of reagents, and so on. By using standard solutions and fixed procedures it is possible to investigate and, consequently, to specify the time for optimum precipitation.

9. Stability of Precipitate

The suspension must be stable for a period of time sufficiently long for making series of optical readings (at least 15 minutes). Not only may the stability of a dispersion often be lengthened by the addition of an increased amount of protective colloid before or after precipitation, but, in addition, the destruction of the precipitating reagent after precipitation is complete may sometimes assist in this direction. Often a suspension which may appear to be unstable insomuch as it rapidly precipitates will, on gentle shaking, readily resuspend to give a reproducible dispersion.

10. Concentration of Reagents

Variations in the concentration of the precipitating reagent often affect considerably the natures of precipitates. For instance, if the precipitate is an organic complex, then the reagent may co-precipitate with it, and consequently modify the optical depth of the resultant suspension. The amount of reagent may also affect the physical nature of the precipitate. Usually excess of a precipitant tends to cause more rapid formation of a coagulum.

11. Adsorption

Amorphous precipitates are particularly prone to adsorb other substances from solution. Thus precipitation of barium sulphate will cause the adsorption from solution of pigments, gums, proteins, as well as of certain inorganic ions. The net result of this adsorption is to vary the nature of the dispersed particles, and so lead to erroneous analytical results. In solutions where this phenomenon may occur, pre-treatment with another adsorbent, e.g. charcoal, may be resorted to provided that it is ascertained that loss of the substance to be estimated does not thereby occur.

12. Co-precipitation

Sometimes when precipitation is induced in a solution, co-precipitation of other substances may result. Again barium sulphate may be taken as an example, for when potassium salts are present these seem to enter into the precipitate in some manner which affects the optical density of the dispersion.

This effect is quite different from the coagulating action of electrolytes on colloids, and must be looked for when investigating variations in precipitation.

13. Complexes

Substances which may form complexes with one or other of the essential reactants will modify to a marked extent the degree and nature of any precipitate. Where it is suspected that such substances may be present, their effects upon standard solutions on precipitation should be investigated.

FLUORIMETRIC TECHNIQUE

FLUORIMETRY is a micro-analytical technique of great sensitivity, and with certain reactions, such as lake formation with beryllium, quantities as low as 0·02 μg. may be detected. This means that a procedure at least a hundred times as sensitive as colorimetry may, at times, be available.

If the concentration of the fluorescent substance in a solution is small and only a minute fraction of the incident radiation is absorbed, then the intensity of the emitted fluorescence is directly proportional to the concentration of the light-emitting substance. At a certain concentration, however,
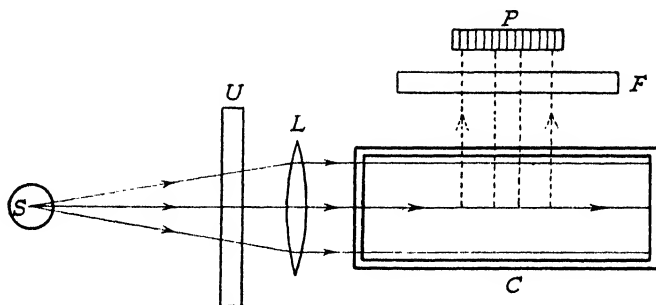
Fig. IV.10.   Diagram of Single Cell Fluorimeter.

S = Mercury vapour lamp.   U = Ultra-violet screen.
L = Collimating lens.   C = Cell containing the solution.
F = Colour filter.   P = Photo-cell.

a maximum fluorescence is usually obtained, above which increase in concentration brings no concomitant increase in fluorescence. It is a good practice, therefore, to dilute a solution progressively and to measure its fluorescence at each dilution.

Fluorimetry is most simply carried out by visual comparison by means of a modified Duboscq colorimeter (see p. 241) used in the same manner as a nephelometer. More accurate results may be obtained, however, if photo-electric instruments are used, as shown in figs. IV.10 and IV.11. When using these instruments, a special filter should be incorporated so that the appropriate wavelength of radiation from an ultra-violet source is used. Maximum fluorescent intensity occurs when the wavelength of

the ultra-violet radiation is identical with that of an absorption maximum in the solution.

Occasionally fluorescence may be due to more than one substance in solution, and in such cases it is often possible to remove the unwanted fluorescence effect by the appropriate use of filters. Errors may sometimes creep in when using these methods on account of the inherent fluorescence of the solvent, in which case blank determinations may be used to neutralise the interfering effect. Aliphatic solvents can usually be employed with
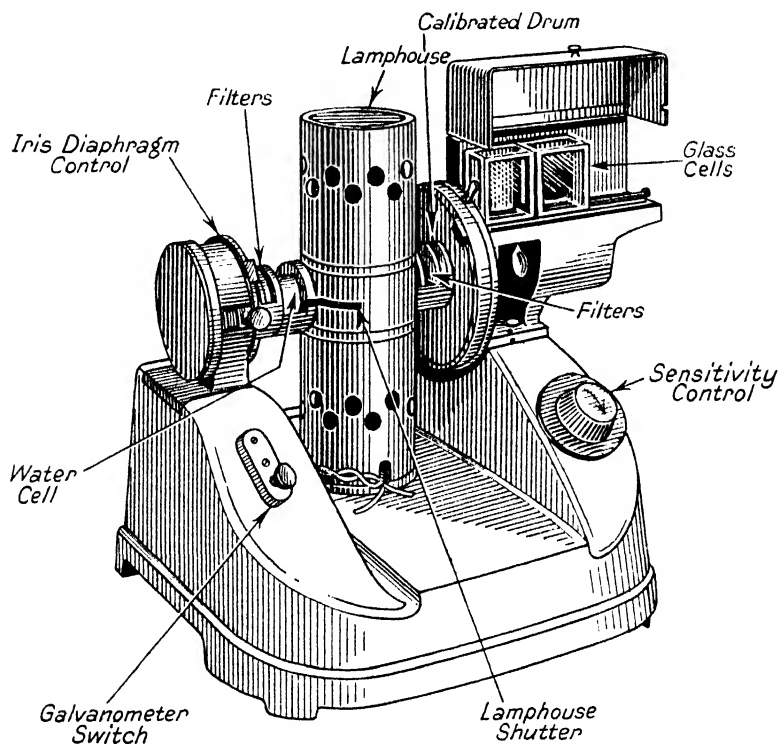
Fig. IV.11. Spekker Photo-electric Fluorimeter.

safety, whereas the majority of aromatic liquids are fluorescent. In general, however, solvents used in fluorimetry should be specially purified by redistillation and inspected before use.

Other substances which may be present in the solution very often have a marked effect upon fluorescence. For instance, chromate ions diminish fluorescence by absorption of the ultra-violet radiation, whilst other substances may interfere by absorbing the emitted light. Glass which has been cleaned in chromic acid solution may therefore affect the accuracy of the result.

Occasionally fluorescent substances are themselves destroyed by the action of ultra-violet light, in which case the reading must be carried out as rapidly as possible.

In addition to taking account of the above considerations, the precautions in technique which have been emphasised in the section dealing with colorimetry may be stressed equally when dealing with fluorimetric methods.

COLORIMETRIC PROCEDURES

THE following pages give in detail a number of colorimetric procedures that can be recommended for accurate micro-analysis. These illustrate the various techniques in standard use.

Many other methods are given in less detail or are indicated in tabular form, references being given to the original papers.

As a whole these procedures deal comprehensively with the determinations of individual substances, inorganic ions, or organic radicals. In addition there have been included (pp. 383–387) methods of group analysis by which a number of different components can be estimated separately in a single analytical sample.

A. METALS

Detailed Procedures

1. COLORIMETRIC ESTIMATION OF COPPER WITH SODIUM DIETHYL-DITHIOCARBAMATE

Example: Estimation of Copper in Foods.*

PRINCIPLES. Foodstuffs are ashed according to recognised methods. The ash is dissolved in nitric acid, evaporated with sulphuric acid to the fuming point, and if a large excess of interfering metals is present, the copper is deposited electrolytically from solution and then redissolved.

The yellow complex with diethyl-dithiocarbamate [viz. $Cu(S—CS—NEt_2)_2$] is formed in a citrate buffer, extracted with carbon tetrachloride, and compared colorimetrically.

The citric acid obviates interference due to small amounts of iron, while turbidities due to the possible presence of traces of aluminium, antimony, bismuth, cadmium, lead, mercury, tin, or zinc will not interfere if the extraction technique is used.

REAGENTS.

1. *Citric acid, 20% solution.*
2. *Ammonia, 10% w/v solution.*
3. *Sodium diethyl-dithiocarbamate, 0·1% aqueous solution, freshly prepared.*

* Haddock, L. A., and Evers, N., *Analyst*, 1932, **57**, 495.

4. *Carbon tetrachloride.*

5. *Anhydrous sodium sulphate.*

6. *Nitric acid, 60% solution.*

METHOD. A portion of foodstuff (e.g. 10 g.) is ashed in a silica crucible, or oxidised by the wet combustion procedure (see p. 100). The ash is then dissolved by warming with 3 ml. of 60% nitric acid, treated with a few drops of concentrated sulphuric acid, and again heated until sulphuric acid fumes appear. The residue is taken up in about 10 ml. of water and neutralised with ammonia.

If grossly contaminated with other metals the acid solution should be transferred to a cell, and the copper separated by electro-deposition (see p. 492). Thereafter the deposited copper can be dissolved in the minimal amount of 50% nitric acid and neutralised with ammonia. However, if iron is not present in the sample to an extent greater than 0·1 mg., then the electro-deposition technique may be omitted.

The neutralised copper solution (*ca.* 10 ml.) is transferred to a small separating funnel and buffered with 10 ml. of 20% solution of citric acid followed by 6 ml. of 10% ammonia. To this mixture are added 10 ml. of a freshly prepared 0·1% solution of sodium diethyl-dithiocarbamate, and, after mixing, 2·5 ml. of carbon tetrachloride. The funnel is stoppered and shaken vigorously. After settling, the carbon tetrachloride layer is run into a dry 10 ml. measure. This extraction procedure is repeated with three further successive 2·5 ml. portions of carbon tetrachloride, and the whole of the extract is united. The final extract should be colourless; if not, successive extractions must be made until all the complex is removed. If the solution is exhausted with four extractions, then the united extract is diluted to 10 ml. with carbon tetrachloride, about 1 g. of anhydrous sodium sulphate is added, and the clear solution is used for colorimetric comparison. If more extractions are necessary, then the final bulk is made up to 20 ml. It was originally suggested that the yellow copper complex should be matched in a Lovibond tintometer against "Lovibond yellow units" but it is more convenient to make the comparison in a colorimeter against standard solutions of copper similarly treated or in a photo-electric instrument previously calibrated for the method.

2. COLORIMETRIC ESTIMATION OF ARSENIC BY FORMATION OF MOLYBDENUM BLUE

Example: Estimation of Arsenic in Foods, Soils, etc.*

PRINCIPLES. Organic matter is destroyed by nitric/sulphuric oxidation (p. 100). Pentavalent arsenic is reduced to the tervalent condition by

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* Milton, R., and Duffield, W., *Analyst*, 1942, **67**, 279.

heating with potassium iodide and stannous chloride. The arsenic is separated by evolution as arsine gas and absorbed in iodine solution, which reoxidises the arsenic to the pentavalent state. The arsenic is finally estimated colorimetrically after reduction of arseno-molybdate to molybdenum blue, using a controlled amount of stannous chloride.

REAGENTS AND APPARATUS.

1. *Arsine generator and absorption-tubes.*

2. *Potassium iodide*, 50% *solution.*

3. *Stannous chloride*, 40% *in concentrated hydrochloric acid.*

4. *Iodine*, 0·02N *solution.*

5. *Sodium bicarbonate, N solution.*

6. *Sodium meta-bisulphite*, 0·5% *solution.*

7. *Sulphuric-molybdate solution.* Mix equal volumes of 13N sulphuric acid with 9·5% sodium molybdate.

8. *Stannous chloride solution.* Dilute 1 part of solution (3) to 200 with water prior to use.

All solutions should be arsenic-free and solutions 4–8 inclusive free from phosphorus also.

METHOD.

1. *Destruction of Organic Matter.* The sample (*ca.* 10 g.) containing arsenic is introduced into a Kjeldahl flask and about 10 ml. of nitric acid are added. The flask is gently heated until solution is obtained, care being taken to avoid charring. Eventually about 2 or 3 ml. of concentrated sulphuric acid are added, with further quantities of nitric acid as required, so as to ensure a steady oxidation without any charring. Digestion is continued until all organic matter is destroyed. To expel excess of nitric acid, the solution is then taken down till the sulphuric acid fumes. The flask is then cooled, diluted with 20 ml. of water, and, in order to destroy nitro-compounds completely, again boiled down until the acid fumes.

2. *Reduction.* The solution in the Kjeldahl flask is diluted to 100 ml. with water so as to reduce the concentration of acid to below 5%, and 1 ml. of 50% potassium iodide solution and 1 ml. of 40% stannous chloride are added. The solution is then brought to the boil to complete reduction of As^v to As^{iii}, and, by maintaining the concentration of acid below 5%, the reduction of H_2SO_4 to H_2S is avoided.

3. *Evolution and Absorption of Arsine.* The arsenite solution, prepared as above, is made up to a known volume and an aliquot portion containing not more than 0·1 mg. of arsenic is transferred to the evolution vessel (see fig. IV.12, *A*). Also introduced into the vessel are 2 ml. of

concentrated sulphuric acid, 10 ml. of concentrated hydrochloric acid, and 1 ml. of 40% stannous chloride. Finally water is added to the 50 ml. mark.

The vessel is stoppered with a rubber bung through the centre of which passes a tube B of about 1·5 cm. diameter and 12 cm. long with a constriction near its middle. The upper part of this tube is loosely packed with glass-wool soaked in lead acetate to absorb the bulk of any hydrogen sulphide which might be evolved and to trap any acid spray. The top of the tube is fitted with a rubber bung from which emerges a capillary tube E of 4 mm. external and 0·5 mm. internal diameter, bent with a double right-angle so that it passes into a narrow adsorption-tube D standing by the side of the evolution vessel. The adsorption-tube is widened at the top so as to prevent loss by splashing, and preferably is graduated at 10 ml.

Into tube D are placed 5 ml. of 0·02N iodine and 1 ml. of N sodium bicarbonate solution. 5 g. of granulated zinc are placed in the evolution vessel A and the stoppers are pressed home. The capillary tube is arranged so that it presses against the bottom of the absorption vessel D, thus ensuring the dispersion of a fine stream of gas bubbles. The evolution is allowed to proceed apace, and under these conditions all the arsine is given off in 30 minutes.

Fig. IV.12.   Arsine Generator.

4. *Colour Development.* The arsine which is absorbed in the iodine solution becomes oxidised to arsenate. Exactly 2·0 ml. of sulphuric-molybdate solution are added, and then exactly 1·0 ml. of 0·5% sodium meta-bisulphite solution, to destroy excess of iodine. After mixing, 1·0 ml. of 0·2% stannous chloride is added, and the blue colour, which is fully developed in a few minutes, is measured in a Spekker absorptiometer (using the red screen).

A calibration curve for the instrument is made by submitting standard solutions of arsenite to the coloration technique which follows:

To 5 ml. of 0·02N iodine and 1 ml. of N sodium bicarbonate in a series of tubes are added 0·005–0·10 mg. of As, then the sulphuric-molybdic solution, and finally metabisulphite solution and stannous chloride, as before.

The method may also be adapted for use with a colorimeter, but due allowance must be made for the reagent blank in this case.

3. COLORIMETRIC ESTIMATION OF ANTIMONY WITH PYRIDINE AND POTASSIUM IODIDE

Example: Estimation of Antimony in Copper Alloys.*

PRINCIPLES. The sample is dissolved in acid, boiled with hypophosphite to separate arsenic, and the antimony is then removed from solution by deposition on copper foil (Reinsch reaction). After re-solution in sodium peroxide and removal of heavy metals with alkali-insoluble sulphides, the antimony is separated as a yellow complex by reduction with iodide and sulphur dioxide in the presence of pyridine. The complex, stabilised with gum acacia, is matched against a standard antimony solution.

REAGENTS.

1. *Hydrochloric acid, 5N.*
2. *Sodium hypophosphite.*
3. *Nitric acid, 5N.*
4. *Sodium peroxide.*
5. *Ammonium nitrate, 10%.*
6. *Gum acacia, 10%.*
7. *Potassium iodide, 20%.*
8. *Pyridine, 10%.*
9. *1/10th saturated aqueous sulphur dioxide.*
10. *Sulphuric acid, 9N.*

11. *Standard antimony solution.* Dissolve 0·267 g. of potassium antimony tartrate in 1 l. of 10% hydrochloric acid.

METHOD. 5 g. of the sample are dissolved in 30 ml. of 9N sulphuric acid and 15 ml. of concentrated nitric acid, and evaporated until sulphuric acid fumes appear. After cooling the residue is treated with 300 ml. of 5N hydrochloric acid, 10 g. of sodium hypophosphite are added, and the mixture is boiled for 10 minutes. If a precipitate forms the boiling is continued for a further 10 minutes to ensure precipitation of all arsenic. The arsenic is then coagulated by shaking the cooled solution with about 20 ml. of benzene and separated by filtration through a wet filter-paper. The filtrate and washings from the precipitate are united and then treated for removal of antimony.

The solution at this stage should contain 20% hydrochloric acid. A copper strip, size 15 cm. × 2 cm., is cleaned with 5N nitric acid, washed, and then rolled into an open flat spiral. This is introduced into the solution, which is then boiled gently for about 2 hours, after which time the solution is poured off and the copper spiral is rapidly rinsed with cold distilled water. Since freshly precipitated antimony is appreciably soluble the washing

* Clark, S. G., and Evans, B. S., *Analyst*, 1929, **54**, 23.

process *must* be carried out rapidly. The spiral is then transferred to a small beaker, covered with distilled water, and 1 g. of sodium peroxide is immediately added. After about 5 minutes the contents of the beaker are warmed until the copper darkens all over, then the liquid is removed and the copper and beaker are washed with water once or twice.

The alkaline antimony solution from the foil together with the washings are now treated for about 15 seconds with a current of sulphuretted hydrogen, and the beaker is placed on a boiling water-bath until the precipitated sulphides of heavy metals are coagulated. This precipitate is removed by filtration, washed with a few millilitres of ammonium nitrate solution, and discarded. The filtrate is treated with concentrated sulphuric acid, taken down to the fuming point, and oxidised completely by addition of a few drops of nitric acid. Excess nitric acid is removed, and the cooled acid solution is diluted to about 15 ml. with water.

10 ml. of 1% gum acacia, 5 ml. of 20% potassium iodide, 1 ml. of 10% aqueous pyridine, 1 ml. of 10% of saturated sulphur dioxide solution, and 60 ml. of 9N sulphuric acid are placed in a measuring cylinder. To this mixture is then added the acid antimony solution, and the whole is diluted to 100 ml. The yellow colour produced is then measured colorimetrically against a standard solution of antimony, which, after treatment with 9 ml. of 9N sulphuric acid, is added to the reagents mixed in the manner named above. The standard should preferably contain not more than 0·1 mg. of antimony, since colour matching is difficult with quantities of the order of 1 mg. of Sb. Moreover, precipitation may then occur.

4. COLORIMETRIC ESTIMATION OF TIN WITH 4-METHYL-1 : 2-DIMERCAPTOBENZENE, "THIOL"

Example: Estimation of Tin in Organic Material.*

PRINCIPLES. Tin is separated from interfering metals by the distillation of stannic bromide and a reddish coloration is then produced with 4-methyl-1 : 2-dimercaptobenzene solution.

Silver, copper, mercury, lead, bismuth, cadmium, arsenic, antimony, nickel, and cobalt react with "thiol" to give coloured precipitates. Of these metals only arsenic is distilled over with the tin in the procedure outlined above. If present in large quantities it should be removed, but small amounts (equal to that of the tin present) may be tolerated.

REAGENTS.

1. *Hydrobromic acid, sp. gr.* 1·46–1·49.
2. *Phenol, 25% solution in glacial acetic acid.*

* Law, N. H., *Analyst*, 1942, **67**, 283.

3. *Thioglycollic acid*, 0·4% *solution*.

4. *Agar mucilage* (gum ghatti is better, see p. 291).

5. *4-Methyl-1 : 2-dimercaptobenzene ("thiol"), 0·1% solution in 1% caustic soda* (freshly prepared).

METHOD. The sample is ignited in a silica crucible to destroy organic matter. To avoid volatilisation losses the temperature should not be beyond dull red heat. The residue is quantitatively transferred to a special all-glass distillation apparatus (fig. IV.13), using about 30 ml. of concentrated sulphuric acid in the process. The distillation flask is fitted with a funnel filled with hydrobromic acid and also contains an inlet tube through which passes a stream of carbon dioxide. The flask is heated in an oil-bath maintained at 220° C. When this temperature is reached, the hydrobromic
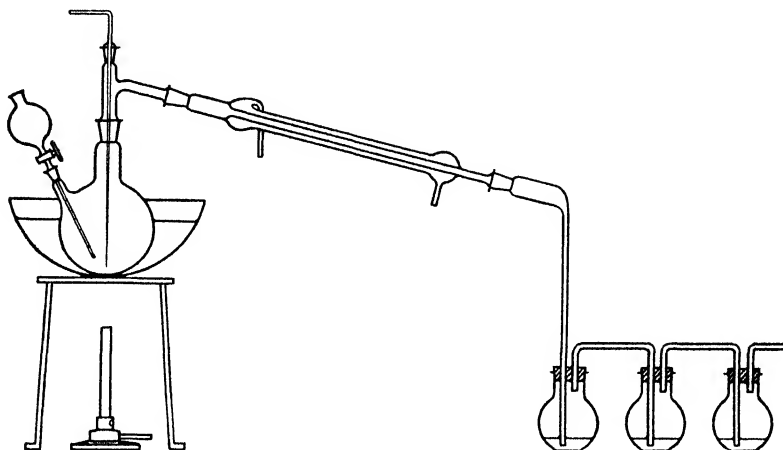
Fig. IV.13.   Micro-distillation of Stannic Chloride.

acid (which is kept cool) is allowed to flow into the flask at the rate of 60 or 70 drops per minute and at the same time a steady stream of carbon dioxide is bubbled through the solution. The distillate is cooled in a water condenser and collected in a series of receiving flasks containing water. Distillation is allowed to proceed for about 1 hour, the condenser rinsed, and the rinsings, together with the combined distillates, are diluted to a known volume, e.g. 50 ml.

An aliquot of this solution is taken and free bromine therein is destroyed by the dropwise addition of 25% phenol in glacial acetic acid.

To determine the strong acid concentration a portion of the solution is then titrated with N caustic soda to methyl orange end-point. A second aliquot is then taken, bromine is again destroyed by addition of phenol, and 30% caustic soda is added (according to the previously determined titration) until residual concentration of free hydrobromic acid is equivalent

to 0·5 ml. of the original hydrogen bromide reagent. Then 0·2 ml. of thioglycollic acid solution, 4 drops of agar mucilage, and 1 ml. of *freshly prepared* thiol reagent (0·1% in 1% NaOH) are successively added, and the mixture is diluted to 10 ml. and heated on a water-bath for 1 minute. After cooling, the colour is compared against a similarly prepared standard solution of tin (e.g. 0·02 mg. Sn) in a colorimeter or in an absorptiometer calibrated for the purpose.

5. COLORIMETRIC ESTIMATION OF LEAD USING DITHIZONE

Example: Estimation of Lead in Bone, etc.*

PRINCIPLES. The substance is subjected to wet oxidation, and lead sulphate, which is apt to be adsorbed in the insoluble calcium sulphate precipitate, is abstracted by conversion to lead carbonate, dissolved in acid, and so brought into solution. The lead ions are extracted with dithizone in the presence of citric acid and cyanide. Colorimetric estimation is made after subsequent oxidation of the lead dithizonate and reprecipitation as the colloidal sulphide.

REAGENTS.

1. *Ammonium acetate, saturated solution.*
2. *Sodium carbonate, 4% solution.*
3. *Sodium cyanide, 10%.*
4. *Purified dithizone in chloroform, 0·1%* (see p. 189).
5. *Hydrogen peroxide, 20 volumes.*
6. *Sodium sulphide, 10% solution.*

METHOD. Wet oxidation with sulphuric and nitric acids is carried out. The solution is diluted with water, transferred to a large centrifuge tube, and centrifuged. The clear liquid is decanted back into the original oxidation flask and a little ammonium acetate solution is added to dissolve any lead sulphate which might be adhering to its walls. The precipitate in the centrifuge tube is washed twice with 20 ml. of hot water and the washings are united with the original solution in the flask. 100 ml. of 4% sodium carbonate solution are added to the precipitate in the centrifuge tube, which is placed in a boiling water-bath for 4 hours and stirred repeatedly.

At the end of this period the tube is again centrifuged and the supernatant liquid is added to the original flask. To this solution are added 5 g. of citric acid, and then ammonia until alkaline, plus a few drops in excess. 1 ml. of sodium cyanide solution is added, and the solution extracted with three successive 10 ml. quantities of 0·1% dithizone in chloroform. The insoluble carbonate residue in the centrifuge tube is dissolved in dilute hydrochloric acid, and the solution is boiled to remove carbon dioxide.

* Roche Lynch, G., Slater, R. H., and Osler, I. G., *Analyst*, 1934, **59**, 787.

Citric acid, ammonia, and cyanide are added as before, and the solution is extracted with dithizone. This extract is mixed with that obtained from the soluble portion, the combined extracts are washed with water, and then the chloroform solution is distilled to dryness. The residue is oxidised with sulphuric acid and hydrogen peroxide, diluted with ammonium acetate solution, made alkaline with ammonia, and cyanide and sodium sulphide are added. The lead sulphide coloration is then compared against standards of lead nitrate which have been treated with ammonium acetate, cyanide, and sodium sulphide in the same way.

If it is suspected that bismuth is present, the above method must be extended to include another separation as lead sulphate after the oxidation of the dithizone extract.

Other Methods: (i) Direct measurement of the dithizone colour,* (ii) electrolytic deposition as PbO_2 followed by oxidation of tetramethyldiaminodiphenylmethane to a blue colour,† and (iii) separation as chromate and estimation of chromate with diphenylcarbazide.‡

6. Colorimetric Estimation of Bismuth

(a) Method using Dithizone.§

PRINCIPLES. The sample is brought to solution, precipitated as the sulphide, redissolved, extracted with dithizone, purified from lead, and estimated colorimetrically.

REAGENTS.

1. *Potassium cyanide*, 10%.
2. *Ammonia*, 50%.
3. *Purified dithizone*, 0·0025% in chloroform.
4. *Ammonia*, 15% solution.
5. *Dithizone*, 0·0006%.

METHOD. The sample is subjected to wet oxidation, nitric acid is removed, and, after neutralisation, the solution is adjusted to $0·3N$ with standard acid. 3 mg. of copper sulphate are added to the solution, and hydrogen sulphide is passed in for 1 hour. The precipitate is centrifuged at high speed and washed with dilute acid saturated with hydrogen sulphide. The precipitate is dissolved in a few drops of hot nitric acid and the bulk of the nitric acid is then removed by evaporation. The residue is diluted to about 50 ml. and transferred to a separating funnel. About 5 ml. of 10% potassium

* Fischer, H., and Leopoldi, G., *Z. Anal. Chem.*, 1940, **119**, 161.
† Muller, H., *Z. Anal. Chem.*, 1938, **113**, 161.
‡ McCarthy, W. J., *Am. Ass. Offic. Agric. Chem.*, 1932, **15**, 370.
§ Hubbard, D. M., *Ind. Eng. Chem.* (Anal. Edn.), 1939, **11**, 343.

cyanide are added, followed by ammonia to bring the mixture to pH 8·5. The solution is now extracted with 5 ml. portions of 0·0025% solution of dithizone in chloroform until the orange colour due to bismuth no longer extracts. 3 ml. of chloroform are then shaken with the aqueous solution and then are added to the combined chloroform extracts. The aqueous solution is re-acidified with nitric acid, again adjusted to pH 8·5 with ammonia, and extracted twice with 5 ml. portions of chloroform. The combined extracts are washed with water and then extracted with two 25 ml. portions of 1% nitric acid solution. The aqueous phase is then adjusted to pH 2·5 with dilute ammonia and thoroughly extracted with 5 ml. portions of dithizone solution to remove bismuth from the aqueous phase (lead not being extracted). The chloroform solution is now treated with two 25 ml. portions of 1% nitric acid. This aqueous solution of bismuth is then treated with 2 ml. of 10% potassium cyanide in 15% ammonia solution, and extracted with 10 ml. of 0·0006% solution of dithizone in chloroform if the bismuth content is less than 5 mg., or with 25 ml. of 0·0012% solution if the bismuth content is between 5 and 25 mg. The optical density of the solution is then measured using light of wavelength 505 mμ and the result is compared against a reference graph constructed from results given by standard solutions of bismuth.

(b) Other Methods: (i) Colorimetric estimation of the orange double iodide of bismuth and potassium,* or (ii) of the yellow thiourea-bismuth complex;† (iii) separation as bismuth tannate and estimation with phospho-tungsto-molybdic acid,‡ or (iv) by means of the red coloration of bismuth o-hydroxy-quinolinate.§

7. COLORIMETRIC ESTIMATION OF ALUMINIUM AS A LAKE WITH AURIN TRICARBOXYLIC ACID

Example: Estimation of Aluminium in Water.‖

PRINCIPLES. The water sample is evaporated to small bulk. Iron is removed by extraction with ether in hydrochloric acid solution, and the aluminium is made to combine with aurin tricarboxylic acid to form a coloured lake.

REAGENTS.
1. *Aurin tricarboxylic acid,* 0·2% *solution of the ammonium salt.*
2. *Ammonium carbonate, saturated solution in sp. gr.* 0·880 *ammonia.*

* Haddock, L. A., *Analyst*, 1934, **59**, 163.
† Tompsett, S. L., *Analyst*, 1938, **63**, 250.
‡ Teitelbaum, M., *Z. Anal. Chem.*, 1930, **82**, 366.
§ Sazeiac, R., and Pourzerques, J., *Compt. rend. Soc. Biol.*, 1932, **109**, 79.
‖ Yoe, J. H., and Hill, W. L., *J.A.C.S.*, 1927, **49**, 2395; Sherrer, J. A., and Morgerman, W. D., *J. Res. Nat. Standards*, 1928, **21**, 105.

METHOD. About 300 ml. of water are evaporated to dryness in a platinum dish, and the residue is ignited at a dull red heat for a few minutes. After cooling about 0·2 ml. of hydrochloric acid is added to the dish and then about 5 ml. of distilled water. The contents of the dish are brought to the boil, quantitatively transferred to a centrifuge tube, and any silica precipitate is removed by centrifuging. The supernatant fluid is then made acid with hydrochloric acid until a concentration of 20% is reached, and ferric chloride is extracted by shaking with an equal volume of water-saturated ether. To ensure the complete removal of iron two successive extractions must be made.

The watery solution is warmed to remove ether and diluted to about 30 ml. with distilled water. 5 ml. of glacial acetic acid are added, followed by 0·2 ml. of aurin carboxylic acid reagent. Ammonia+ammonium carbonate reagent is now added, drop by drop, from a burette, with shaking between additions, until a piece of litmus paper dipped into the solution just changes colour. 5 ml. of glacial acetic acid are now added, and an interval of 10 minutes is allowed to elapse. The amount of ammonium carbonate reagent to exactly neutralise the acid is then added, followed by an excess of 5 ml., which is run in slowly with stirring. This double neutralisation is necessary in order to obtain a reproducible coloured lake, since this varies in composition according to the amount of alkali and the manner in which it is added.

The pink colour is matched against standards similarly prepared, measured in an absorptiometer, and the result is referred to a calibration curve. A standard solution may be obtained by dissolving 1·759 g. of potassium alum $[K_2SO_4, Al_2(SO_4)_3, 24H_2O]$ in 1 l. of 1% H_2SO_4 solution. 1 ml. of this solution contains 0·1 mg. of Al. Portions of this solution are diluted ten times and a series of tubes are set up containing 1–10 ml. of this diluted solution. 5 ml. of concentrated hydrochloric acid are added to each tube and then distilled water up to 30 ml. The aurin lake is formed in the manner described above, and the colour depth is measured in a Spekker absorptiometer. A graph relating drum reading to concentration is then constructed. For use with a colorimeter it is advisable to make a standard close to the anticipated concentration.

8. COLORIMETRIC ESTIMATION OF IRON WITH o-PHENANTHROLINE

Example: Estimation of Iron in Organic Material.*

PRINCIPLES. Organic material is destroyed by wet oxidation, to avoid losses either as volatile chloride or by formation of insoluble ferric silicates.

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* Sandell, "Colorimetric Determination of Traces of Metals," p. 276 (Interscience Publishers, New York, 1944); Fortune, W. B., and Mellon, M. G., *Ind. Eng. Chem.* (Anal. Edn.), 1938, **10**, 60.

The iron in solution is reduced with hydroquinone and then allowed to react with *o*-phenanthroline. The red colour is measured colorimetrically.

REAGENTS.

1. *Ammonia, 50% solution by volume.*

2. *Sodium acetate buffer.* Dissolve 100 g. of crystalline sodium acetate and 400 ml. of glacial acetic acid and dilute to 1 l. with water.

3. *Hydroquinone solution.* Dissolve 0·29 g. of hydroquinone in 100 ml. of acetate buffer as required for immediate use.

4. *o-Phenanthroline.* Dissolve 0·29 g. of *o*-phenanthroline monohydrate in 100 ml. of warm water.

METHOD. A sample containing iron of the order of 20–200 μg. is transferred to a Kjeldahl flask and heated with a mixture of 5 parts of nitric and 2 parts of sulphuric acid until the sulphuric acid fumes. More nitric acid is added, a little at a time, until all organic matter is destroyed. Nitro bodies are next removed by diluting the solution with about 20 ml. of water and boiling down to the fuming stage, repeating this whole procedure once.

After cooling, the oxidate is transferred quantitatively to a 25 ml. volumetric flask and made up to the mark with water. 10 ml. of this solution are titrated with 50% ammonia solution until with methyl orange the colour just changes to yellow. A further 10 ml. of the solution are transferred to another tube, the same amount of ammonia solution is added to it, then 5 ml. of buffered hydroquinone+sodium acetate solution and finally 1 ml. of 0·5% *o*-phenanthroline solution. The volume of solution is completed to 25 ml. and the tube is set aside for 1 hour before measuring the intensity of the red colour.

A standard graph for absorptiometric work may be obtained as follows: 0·2159 g. of ferric alum [$K_2SO_4,Fe_2(SO_4)_3,24H_2O$] is dissolved in 1 l. of water. This solution contains 25 mg. of Fe per millilitre. A series of tubes are set up containing from 1–10 ml. of this solution, the acidity is adjusted and the colour is developed in the manner described above. For comparison purposes this will give a range of from 0·025–0·25 mg. of iron.

9. COLORIMETRIC ESTIMATION OF CHROMIUM AS CHROMATE USING DIPHENYLCARBAZIDE

Example: Estimation of Chromium in Silicate Rocks.*

PRINCIPLES. The siliceous material is fused with sodium carbonate and leached with water. Vanadium, which may interfere with the result if

* Sandell, E. B., *Ind. Eng. Chem.* (Anal. Edn.), 1936, **8**, 336.

present in large concentrations, is removed by separation as the oxine and extracted with chloroform. The watery solution is then treated with diphenylcarbazide, which produces an intense violet coloration with chromates.

REAGENTS.

1. *Sulphuric acid, 2N.*

2. *8-Hydroxyquinoline, 2·5% in 2N acetic acid.*

3. *Diphenylcarbazide, 0·25% solution in 50% acetone solution.*

METHOD. About 0·5 g. of the finely ground sample is mixed with five times its weight of sodium carbonate and fused in a platinum crucible for about 20 minutes. About 5 ml. of water are added to the crucible; this causes the fused product to separate as a cake. The whole is then transferred to a small beaker and the melt is broken up with a flat-bottomed glass rod. More water is added and all is dissolved. A few drops of alcohol are now added, and the solution is heated to reduce any manganate which may be present. The solution is filtered into a 25 ml. volumetric flask, and, after washing the filter-paper two or three times with dilute sodium carbonate solution, is diluted to 25 ml. with water. 5 ml. of this solution are taken and titrated with 2N sulphuric acid until the colour of methyl orange indicator passes from yellow to just orange.

An aliquot is then taken and, if vanadium is absent, is treated with 2N sulphuric acid until the solution is 0·2N and the colour is developed as described below (last para.). In the presence of vanadium the aliquot is transferred to a small separating funnel and 0·1 ml. of 8-hydroxyquinoline solution is added. After mixing, the solution is extracted twice with 3 ml. portions of pure chloroform, shaking each time for about 1 minute and discarding the chloroform extract. A further 0·1 ml. of 8-hydroxyquinoline is then added and the extraction with chloroform is again carried out; if all vanadium is extracted, then the last chloroform washing is almost colourless.

To remove chloroform droplets the solution is then filtered through a small wetted paper into a 25 ml. flask. The separating funnel and filter are washed, the washings being added to the flask.

To develop the chromate colour sufficient 2N sulphuric acid to make the solution 0·2N and then 0·1 ml. of 0·25% diphenylcarbazide solution are added, and the contents of the flask are diluted to volume. The violet colour can conveniently be measured by direct visual comparison by transferring the solution to a Nessler glass and adding to a second glass an equivalent volume of 0·2N sulphuric acid plus 0·1 ml. of diphenylcarbazide solution. 0·001N potassium dichromate solution is titrated into the comparison tube from a micro-burette until the two tubes are matched.

10. COLORIMETRIC ESTIMATION OF NICKEL WITH DIMETHYL-GLYOXIME

Example: Estimation of Nickel in Steel.*

PRINCIPLES. The metal is dissolved in hydrochloric and nitric acids, and iron is precipitated by alkaline cyanide, thus maintaining nickel in solution. Copper is then removed as the sulphide in acid solution and the nickel is then estimated by means of the red colour given with dimethyl-glyoxime in the presence of an oxidising reagent (compare p. 44).

REAGENTS.

1. *Ammonia, 50% solution* (by volume).

2. *Potassium cyanide, 1%.*

3. *Hydrochloric acid, 5%.*

4. *Ammonium chloride, 5%.*

5. *Dimethyl-glyoxime, saturated solution in alcohol.*

6. *Sodium hypochlorite, 15%.*

METHOD. In a small beaker covered by a watch-glass about 1 g. of the steel is treated with 10 ml. of concentrated hydrochloric acid and 5 ml. of nitric acid.

The beaker is transferred to a hot plate for a few minutes, to assist dissolution of the metal, and the solution is diluted with distilled water and, after cooling, transferred quantitatively to a 200 ml. volumetric flask. 50% ammonia solution is run in from a burette, with shaking between additions, until there is a slight persistent precipitate of ferric hydroxide, then 2 ml. of 1% potassium cyanide, and, after shaking, 10 ml. of dilute ammonia are added and the flask is filled to the mark with distilled water and its contents are thoroughly mixed.

The ferric hydroxide is then removed by filtration, and an aliquot of the filtrate (to contain not more than 0·1 mg. of nickel) is then treated as follows for removal of copper. The filtrate is neutralised with concentrated hydrochloric acid, which is added in excess to a concentration of about 5%. The solution is warmed and hydrogen sulphide is passed in for about 15 minutes. While still warm, the solution is filtered from precipitated copper sulphide through a paper pulp pad.† The precipitate is washed with 5% ammonium chloride solution and the washings are added to the filtrate. To the filtrate are added 50 ml. of nitric acid, the solution is boiled down to small bulk, evaporated carefully to dryness, and the residue is finally fused so as to remove all ammonium salts by decomposition of the nitrate.

* Jones, B., *Analyst*, 1929, **54**, 582.
† A little paper pulp added to the solution prior to filtration assists in coagulation of the precipitate.

The residue, which should not be over-heated, is then dissolved in a little $N/10$ acid, boiled for a few minutes, transferred to a 100 ml. Nessler glass, and diluted to about 90 ml. with distilled water. 50% ammonia is now added to neutralise, followed by 0·3 ml. in excess. The contents of the tube are now completed to 100 ml., 2 ml. of saturated solution of dimethyl-glyoxime are added, and, after stirring, 1 ml. of sodium hypochlorite solution. The intense red colour is now measured, either in a colorimeter against standards similarly prepared, or with a photo-electric instrument previously calibrated for the method, or by direct visual comparison in a Nessler tube.

A standard solution of nickel is prepared by dissolving 3·368 g. of nickel ammonium sulphate crystals in 1 l. of water and diluting a sample ten times to give a solution containing 0·05 mg. of Ni per millilitre. If the direct visual method is made, then a second Nessler tube is filled to the mark with water, 0·3 ml. of 50% ammonia is added, followed by 2 ml. of dimethyl-glyoxime and after mixing, 1 ml. of hypochlorite. Standard nickel solution is now run in from a burette until the colour depths in the two tubes match.

As alternatives it may be noted:

(*a*) that the water-soluble sodium salt of dimethyl-glyoxime is now available commercially;

(*b*) that the red complex of divalent nickel with dimethyl-glyoxime may be separated by filtration, dissolved in pyridine, and estimated colori-metrically.*

11. COLORIMETRIC ESTIMATION OF COBALT WITH α-NITROSO-β-NAPHTHOL

Example: Estimation of Cobalt in Organic Material.†

PRINCIPLES. After removal of interfering metals by standard precipitation methods, the cobalt, in buffered citrate solution, is treated with α-nitroso-β-naphthol in the presence of a protective colloid. The orange-red colour, which is proportional to the cobalt content, is then measured.

REAGENTS.

1. *α-Nitroso-β-naphthol.* Dissolve 0·1 g. of solid reagent in 20 ml. of $N/20$ caustic soda. Bring to boil, cool, filter, and complete to 100 ml. with water.

2. *Ammonium citrate.* Melt 500 g. of citric acid with 250 ml. of water, and then add 500 ml. of ammonia of sp. gr. 0·880.

3. *Gum ghatti solution.* 5 g. of the gum are suspended in a muslin bag at the top of a cylinder filled with 250 ml. of water and allowed to stand over-night.

* Passamaneck, E., *Ind. Eng. Chem.* (Anal. Edn.), 1945, **17**, 257.

† Jones, E. G., *Analyst*, 1918, **43**, 317.

METHOD. Organic material containing cobalt is ashed in a platinum crucible, and the residue is lixiviated with 5 mg. of 1 : 1 hydrochloric acid. The residue is treated with 4 ml. of 3 : 1 hydrochloric/nitric acid mixture, and again heated. The mixture is then diluted and filtered free from any siliceous residue. The combined solutions are then evaporated to dryness, taken up in dilute hydrochloric acid, and again evaporated to dryness to remove all traces of nitric acid. The residue is then dissolved in 25 ml. of 10% hydrochloric acid. Copper is removed from the solution by saturation with hydrogen sulphide and filtration. The filtrate, after removal of heavy metal precipitates, is evaporated down to remove hydrogen sulphide completely.

Nickel is removed by buffering the solution with excess of ammonium citrate solution and adding a slight excess of a 1% alcoholic solution of dimethyl-glyoxime. Ammonia is added until the solution is slightly alkaline and the precipitate of nickel glyoxime is removed by filtration. The filtrate is evaporated to dryness, ignited, and the residue is taken up in 25 ml. of 10% hydrochloric acid. Manganese is next removed by adding 25 ml. of 50% nitric acid, followed by 0·1 g. of sodium bismuthate, and digesting until manganese is completely precipitated as the brown dioxide.

The solution is filtered, the filtrate and washings are evaporated to dryness, heated with hydrochloric acid to remove nitric acid, and the final residue is taken up in 25 ml. of 50% hydrochloric acid.

To this solution are added 10 ml. of the citrate solution and then, after dilution to 90 ml. with distilled water, 5 ml. of gum ghatti solution followed by 5 ml. of α-nitroso-β-naphthol reagent. The red colour which appears almost immediately is then compared colorimetrically against standard cobalt solutions which have been prepared similarly.

A suitable standard may be made by dissolving 0·4936 g. of cobalt nitrate $[Co(NO_3)_26H_2O]$ in 1 l. of water. Each millilitre will contain 0·1 mg. of Co.

12. COLORIMETRIC ESTIMATION OF MANGANESE AS PERMANGANATE

Example: Estimation of Manganese in Organic Material.*

PRINCIPLES. Organic matter is destroyed by ashing, chlorides are removed by fuming with sulphuric acid, the effect of iron is eliminated by addition of phosphate, and the solution is finally treated with periodate to oxidise manganese to permanganate, which is then measured optically.

REAGENTS.
1. *Sulphuric acid, 12N.*
2. *Syrupy phosphoric acid, sp. gr.* 1·75.
3. *Solid potassium periodate.*

* Richards, M. B., *Analyst*, 1930, **55**, 554.

METHOD. A weighed amount of material (e.g. about 10 g. of foodstuffs or organs) is transferred to a silica crucible and incinerated at a low red heat. The residue is taken up in concentrated hydrochloric acid and, after evaporation, is treated with a few drops of sulphuric and nitric acids. After oxidation of all organic matter is complete, the mixture is again evaporated until white fumes appear. The residue is then treated with 2 ml. of phosphoric acid plus 0·5 ml. of 12N sulphuric acid, and taken down to the fuming point again to ensure removal of all chlorides. After cooling, the solution in the crucible is diluted with water, quantitatively filtered, and the filtrate is taken down to about 10 ml. The solution, which is now at the correct acid concentration for optimum oxidation, is treated with 0·3 g. of potassium periodate, brought to the boil, and maintained in a boiling water-bath for 30 minutes. In this way any manganese is oxidised to permanganate and the resultant colour can be measured.

Standard solutions of manganese are prepared by dissolving 0·144 g. of potassium permanganate in 100 ml. of water, decolorising with sulphur dioxide, and boiling until excess of sulphur dioxide is removed. 20 ml. of this solution are then diluted to 1 l. with 2N sulphuric acid. This solution will contain 0·001 mg. of Mn per millilitre, and is used for comparison standards after being boiled with periodate in the manner described above.

13. COLORIMETRIC ESTIMATION OF CALCIUM VIA CALCIUM PHOSPHATE AND MOLYBDENUM BLUE

Example: The Micro-estimation of Calcium in Pus.*

PRINCIPLES. Calcium is precipitated as the insoluble phosphate. The phosphorus in the precipitate is then measured colorimetrically after coupling with molybdic acid and reduction of the product to molybdenum blue.

REAGENTS.

1. *Phosphate solution.* 1 g. of tri-sodium phosphate is dissolved in 50 ml. of water and mixed with 50 ml. of 20% caustic soda. The solution is allowed to stand over-night and any precipitate is then centrifuged off.

2. *Trichloracetic acid, 7% solution.*

3. *Alcohol, 50%, made faintly alkaline with a drop of ammonia.*

4. *Molybdic acid solution.* Equal volumes of sodium molybdate 7·5% and 10N sulphuric acid are mixed.

5. *Stannous chloride solution.* 5 g. of stannous chloride are dissolved in 10 ml. of concentrated hydrochloric acid. This solution is diluted 1/200 with water when required.

* Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, 1927, **7**, 517.

METHOD. 0·10 ml. of pus is transferred to a platinum dish *via* a 0·2 ml. pipette. The pipette is rinsed with water and the rinsings are added to the dish. The contents of the dish are gently evaporated and ignited to remove all organic matter. The residue is then dissolved in 0·25 ml. of 7% trichloracetic acid solution, and, with the aid of a small capillary pipette, the solution is transferred to a small centrifuge tube. The crucible is rinsed with three further 0·25 ml. quantities of trichloracetic acid and the rinsings are also transferred to the centrifuge tube by means of the small pipette. 0·2 ml. of the alkaline phosphate solution is then added and the contents of the tube are mixed. After standing aside for 1 hour, in order to allow the precipitate of calcium phosphate to settle, the tube is centrifuged at high speed and the supernatant fluid is removed by decantation. The precipitate is broken up by tapping both the bottom and the sides of the tube, and is washed with 1 ml. of alkaline 50% alcohol. After centrifuging and removal of the wash liquid, the washing process is repeated, but this time the tube is drained by inversion over filter-paper for a few minutes (see p. 34). The lip of the tube is finally dried by touching with a filter-paper.

The precipitate in the tube is then dissolved in 0·20 ml. of the molybdic acid reagent and 0·70 ml. of water is then added. This solution is then treated with 0·10 ml. of diluted stannous chloride solution, bringing the volume in the tube to 1·0 ml.

The blue colour which immediately develops is then compared in a micro-colorimeter against standard solutions of calcium which have been pre-cipitated and treated in a similar manner. A convenient solution of calcium for this purpose contains 10 mg. of Ca per 100 ml.

14. COLORIMETRIC ESTIMATION OF MAGNESIUM BY USE OF 8-HYDROXY-QUINOLINE*

PRINCIPLES. After suitable preparation of the sample for analysis, magnesium is separated as the salt of 8-hydroxy-quinoline. The precipitate is dissolved and coupled with diazotised sulphanilic acid and the dyestuff is measured optically.

Manganese and zinc may interfere with this procedure and, if present, must be removed, together with iron and aluminium, by the alkaline sulphide technique. In this case ammonium salts should be removed before proceeding with the oxine separation.

Other metals which yield insoluble oxinates (see Part I, p. 44) may be estimated colorimetrically in the same way.

* Alten, F., Weiland, H., and Kurmies, B., *Angew. Chem.*, 1933, **46**, 697.

REAGENTS.

1. *Sodium acetate, saturated solution.*

2. *8-Hydroxy-quinoline.* Dissolve 4 g. in 8 ml. of glacial acetic acid and pour into 200 ml. of hot distilled water.

3. *Sodium oxalate, saturated solution.*

4. *Sodium tartrate, saturated solution.*

5. *Ammonia,* 50% *w/v.*

6. *Hydrochloric acid N.*

7. *Sulphanilic acid, M/200 solution in* 30% *acetic acid.*

8. *Sodium nitrite,* 0·3% *solution, freshly prepared.*

METHOD. The sample, if organic in nature, is ashed in a silica crucible or is decomposed by the wet oxidation procedure (see p. 100). The residue is taken up in dilute hydrochloric acid and heavy metals are precipitated by passing a current of hydrogen sulphide through the solution. Excess hydrogen sulphide is removed from the filtrate, which is then evaporated to small bulk and made up to a known volume. A portion of the solution, to contain about 0·01–0·02 mg. of magnesium in about 1 ml., is buffered with sodium acetate solution, 0·5 ml. of which is added in excess. 0·5 ml. of hydroxy-quinoline reagent is then added and the mixture is stood aside for 3 hours in order to complete the precipitation of any iron or aluminium which might be present. After heating, 0·2 ml. of saturated sodium oxalate is added and the solution is maintained at the boiling-point for about 30 minutes. The oxine precipitates of iron and aluminium, together with the oxalate precipitate of calcium, are centrifuged down together. The supernatant liquid is quantitatively transferred to another centrifuge tube, the precipitate is washed with 1 ml. of cold water, and, after centrifuging, the wash liquid is added to the original supernatant liquid, whilst the residual precipitate is discarded. 1 ml. of saturated sodium tartrate, 0·3 ml. of oxine reagent, and 1 ml. of 2N caustic soda are added to the combined liquid, which is stood for some hours, preferably over-night, and then held at boiling-water temperature for about 30 minutes prior to centrifuging.

The precipitate thus separated is washed twice with 2 ml. of 2% ammonia solution to remove excess oxine, and then dissolved in 5 ml. of hot hydrochloric acid and transferred quantitatively to a 50 ml. graduated flask, using about 30 ml. of water. To the flask is now added 0·5 ml. of sulphanilic acid solution followed by 0·5 ml. of freshly prepared sodium nitrite solution. After an interval of 15 minutes, to allow for coupling of the dyestuff, the solution is made alkaline by addition of 10 ml. of 2N caustic soda and completed to 50 ml. with distilled water. The red colour is then measured by optical methods.

A standardisation of the method must be made, using magnesium solutions which have been subjected to the precipitation procedure outlined above,

since colorations obtained with 8-hydroxy-quinoline directly are differents in tone from those given by magnesium oxine solutions. A standard is conveniently prepared by dissolving 1·014 g. of $MgSO_4,7H_2O$ in a litre of water and diluting this solution five times prior to use. This solution will contain 0·02 mg. of magnesium per millilitre. For colorimetric comparison, 0·5 ml. of this solution is treated by the precipitation procedure and the colour is developed as above.

If a photo-electric end-point is used, then volumes of 0·2, 0·4, up to 1·0 ml. of the solution are used to prepare for calibration an optical density/concentration graph of range from 0·004 mg. to 0·02 mg. of magnesium.

15. COLORIMETRIC ESTIMATION OF SODIUM BY USE OF URANYL ACETATE

Example: Estimation of Sodium in Foods.*

PRINCIPLES. Organic matter is destroyed by wet oxidation, potassium is removed as perchlorate, and phosphate as the barium salt. Sodium is precipitated as the uranyl magnesium salt and estimated colorimetrically after solution and formation of brown uranyl ferrocyanide.

REAGENTS.

1. *Magnesium perchlorate, saturated solution in alcohol.*

2. *Alcohol, 95%.*

3. *Barium chloride, 5%.*

4. *Ammonia, 1% solution.*

5. *Acetic acid, 10%.*

6. *Sodium precipitating reagent.* 7 g. of uranium acetate are ground into 100 ml. of 21% w/v of anhydrous magnesium acetate and allowed to stand over-night. Filter before use.

7. *Ferrocyanide reagent.* 10 g. of gum ghatti are dissolved in 500 ml. of water by allowing to suspend over-night in a muslin bag at the top of a cylinder filled with water. After filtration 5 g. of potassium ferrocyanide are dissolved in the solution.

METHOD. The sample of dried food (e.g. 1 g.) is oxidised by the wet procedure using ammonium nitrate/nitric acid mixture (see p. 101). The residue is boiled with dilute hydrochloric acid to hydrolyse metaphosphates and the solution is made up to a known volume (e.g. 10 ml.).

An aliquot (e.g. 4 ml. corresponding to 0·4 g. of foodstuff) is pipetted into a centrifuge tube and to this is added 1 ml. of saturated magnesium perchlorate. After standing for 30 minutes the tube is centrifuged to

* McCance, R. A., and Shipp, H. L., *Biochem. J.*, 1931, **25**, 449; Milton, R., Hoskins, J., and Jackman, W., *Analyst*, 1944, **69**, 299.

separate the precipitate of potassium perchlorate. 2 ml. of the super-
natant solution are transferred to another tube. Phosphates are eliminated
by adding 1 ml. of 5% barium chloride and then 1 ml. of 1% ammonia
solution. After mixing, the tube is again centrifuged to throw down any
barium phosphate. 2 ml. of the supernatant fluid from this tube are
transferred to another centrifuge-tube, and 1 ml. of 10% acetic acid and
7 ml. of sodium precipitating reagent are added. The tube is stood aside
for 1 hour to complete precipitation of the sodium salt, and then is centri-
fuged. The supernatant fluid is removed and the precipitate is washed
twice with 2 ml. of 95% alcohol, carefully draining off the liquid between
washings.

The precipitate is now dissolved in 10·0 ml. of distilled water. 1·0 ml.
of this solution is transferred to a colorimeter tube, to which are added
4·0 ml. of water and then 5·0 ml. of ferrocyanide reagent. The clear brown
colour is then measured in a photo-electric absorptiometer or is compared
against standards in a colorimeter.

A calibration curve may be prepared as follows: 254 mg. of sodium
chloride are dissolved in 100 ml. of distilled water. 100 ml. of sodium
reagent are added and, after standing for 1 hour, the precipitate is filtered
through a sintered glass crucible and washed free from excess reagent with
95% alcohol. It is then dissolved in 100 ml. of water, giving a solution
equivalent to 1 mg. of Na per millilitre. A portion of this solution is then
diluted twentyfold, and measured portions of from 1-5 ml. are pipetted
into a series of tubes. The volume in each tube is made up to 5·0 ml. with
water, and to each tube are added 5·0 ml. of ferrocyanide reagent. The
colours produced, giving a range from 0·02–0·10 mg. of Na are then
measured in a Spekker absorptiometer and drum reading is plotted against
concentration.

16. COLORIMETRIC ESTIMATION OF AMMONIA IN WATER USING
NESSLER'S REAGENT*

(a) Free and Saline Ammonia.

PRINCIPLES. The term "free and saline" is applied to all ammonia which is
liberated by distillation with very dilute alkali. The distillate is treated
with Nessler's solution and the colour is matched against standards.

REAGENTS.

1. *Nessler's reagent* (according to Wanklyn and Chapman). Dissolve
70 g. of potassium iodide in 400 ml. of water and add 500 ml. of saturated
(5%) mercuric chloride solution, with stirring, until the scarlet precipitate
is just permanent. Then stir in a solution of 240 g. caustic soda in 500 ml.

* Dickenson, D., "Chemical Analysis of Water" (Blackie and Son, 1944), p. 36.

of water, and make up to 2 l. with distilled water. Add a little more mercuric chloride solution until there is a permanent turbidity. On the following day decant the mixture into a dark bottle.

2. *Ammonia-free distilled water.* To obtain this treat a quantity of water with a little sulphuric acid and a crystal of permanganate, and distil into a clean glass vessel. The distillation should not be carried on too far.

METHOD. A sample of water, e.g. 500 ml. of tap-water, is distilled from an all-glass distillation apparatus and about 350 ml. of distillate in all is collected. 50 ml. of this are transferred to a Nessler glass. To similar Nessler glasses are added 49 ml. of ammonia-free distilled water and 1 ml. of standard solutions of ammonia (0·002 mg.–0·02 mg.). After mixing, 2 ml. of Nessler solution are added to each tube and the aliquot distillate is compared visually against standard tubes.

The use of dilute alkali to liberate ammonia during distillation is usually unnecessary in view of the slight alkalinity of the glass distillation apparatus.

(b) Albuminoid Ammonia.

PRINCIPLES. "Albuminoid ammonia" is the term used to describe the ammonia which is liberated from organic matter by oxidation by permanganate in strongly alkaline solution.

REAGENT.

Alkaline permanganate. Equal volumes of 0·8% potassium permanganate and 30% caustic soda are mixed and evaporated to half bulk.

METHOD. Ammonia-free distilled water is added to the residue in the distillation flask after removal of free ammonia, until the total volume is about 400 ml. 35 ml. of alkaline permanganate solution are then added, and the distillation is resumed. About 250 ml. of distillate are collected and 50 ml. of this are transferred to a Nessler tube, 2 ml. of Nessler solution are added, and after 2 minutes the colour is compared with standard solutions of ammonia.

OTHER COLORIMETRIC METHODS FOR METALS

17. ESTIMATION OF GOLD*

PRINCIPLES. Auric salts in acid solution oxidise o-tolidine to a yellow dyestuff which may be measured. Free chlorine, nitrous acid, and certain other oxidisable metals also give a yellow colour, and the concentration of mineral acid should not be more than $0.1N$. Tellurium is used as a "collector" in the initial separation (compare p. 259).

The method will estimate gold down to 0.5 part per million.

METHOD. To the solution containing gold is added about 0.5 g. of sodium tellurite and then strong hydrochloric acid until the acid concentration in the solution is about 10%. Sulphur dioxide is passed in until the tellurium is precipitated as a colloidal solution. The solution is warmed to coagulate the tellurium and is filtered through a small ashless paper. The paper and precipitate are transferred to a platinum basin and ignited to destroy the paper and to volatilise the tellurium. The residue is dissolved in chlorine water and the excess of free chlorine is removed completely by passing a stream of air through the solution. The solution is then made acid with hydrochloric acid equivalent to $0.1N$ and 1/25th of its volume of 0.1% o-tolidine in N hydrochloric acid is added. The yellow colour is matched against standards, or is measured in an absorptiometer.

Other methods which can be used are:

Reduction with hydroquinone,† reduction with stannous chloride,‡ reduction with formaldehyde,§ and combination with dimethylaminobenzal-rhodanine.‖

18. ESTIMATION OF SILVER ¶

PRINCIPLES. Silver ions in solution give a red colour with p-dimethyl-aminobenzylidine-rhodanine which is specific only in the absence of gold, platinum, palladium, cuprous and mercurous salts (compare Part I, p. 49).

METHOD. To the silver solution containing from 1–100 μg. of Ag in 10 ml. of water are added 1 g. of ammonium acetate, then 0.5 ml. of an 0.2% alcoholic solution of p-dimethylaminobenzylidine-rhodanine solution, and, after

* Pollard, W. B., Analyst, 1919, 44, 94.
† Beamish, F. E., Russell, J., and Seath, J., Ind. Eng. Chem. (Anal. Edn.), 1937, 9, 137.
‡ Brodigan, C., Met. Chem. Eng., 1914, 12, 460.
§ Muller, A., and Foix, A., Bull. Soc. Chim., 1922, 31, 717.
‖ Merejkovsky, B., Bull. Soc. Chim. Biol., 1933, 15, 1336.
¶ Feigl, F., Z. Anal. Chem., 1928, 74, 380.

mixing, the red colour is measured, preferably in a photo-electric absorptiometer. In the preparation of the standard solution it is best to keep a strong solution of silver nitrate and dilute as required, since silver ions are adsorbed on to the glass vessel used as a container (see p. 268).

Another method is reduction to colloidal silver with hydrosulphite.*

19. ESTIMATION OF MERCURY†

Example: Estimation of Traces of Mercury in the Atmosphere.

PRINCIPLES. Air containing mercury is aspirated through an absorption bubbler containing sodium hypobromite. Mercury vapour is trapped and fixed as the bromide. Excess of hypobromite is destroyed with hydroxylamine and the mercury is then extracted as the coloured complex with dithizone and estimated colorimetrically. The method will readily measure 5 μg. of mercury in solution.

REAGENTS.

1. *Hypobromite solution.* 4 ml. of bromine are dissolved in 100 ml. of an 8% solution of sodium hydroxide.

2. *Hydroxylamine hydrochloride, 50% solution.*

3. *Hydrochloric acid, 10% solution.*

4. *Dithizone, 0·005% solution.*

5. *Ammonia, 10% solution.*
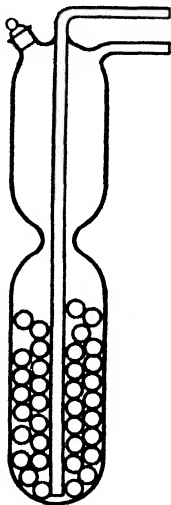
Fig. IV.14.
Absorption Apparatus for Collecting Impurities in Air.

METHOD. A bead bubbler (see fig. IV.14) is charged with 10 ml. of sodium hypobromite solution and attached to a source of suction having an airflow meter in the circuit. Air is drawn through the system at a given rate (e.g. 10 l. per minute) for a given time period (e.g. 100 minutes).

The contents of the bubbler are transferred quantitatively to a flask and made just acid with hydrochloric acid. An aqueous solution of hydroxylamine is then added, drop by drop, until the brown colour due to bromine is discharged. A small piece of litmus paper is introduced into the solution and caustic soda is added until the mixture is just neutral. Hydrochloric acid is then added until the solution is at pH 1·0. The solution is then extracted with several successive portions of very weak dithizone solution

* Jelley, E. E., *J. Soc. Chem. Ind.*, 1932, **51**, 191–3T.
† Milton, R., and Duffield, W. D., *Analyst*, 1947, **72**, 11.

until the extracts are no longer purple. The combined extracts are then extracted several times with 5% ammonia in order to remove excess dithizone and to change the mercury complex to the keto form. The resulting brown colour is measured against standards in a Spekker absorptiometer.

20. ESTIMATION OF PLATINUM*

Example: Determination of Platinum Salts and Metal in Refinery Atmosphere.

PRINCIPLES. The air is drawn through a particulate filter, e.g. a No. 43 Whatman paper (see fig. III.19 on p. 190). The filter and the substances collected upon it are digested with aqua regia and brought into solution. The diluted solution is treated with thallium nitrate, which forms a thallium complex of the precious metals. On treatment with ammonia the complexes of all metals, except platinum, will dissolve and are removed. The platinum complex is dissolved with acid, and on reduction to the platinous state by the addition of stannous chloride produces an orange colour suitable for colorimetric estimation.

REAGENTS.

1. *Aqua regia* (2 parts hydrochloric—1 part nitric).
2. *Thallium nitrate, 1% solution.*
3. *Hydrochloric acid, 3% by volume.*
4. *Stannous chloride, 4% solution in hydrochloric acid.*

METHOD. The dust is collected by the method described on p. 190, and the filter-pad containing the dust sample is then placed in a Kjeldahl flask and thoroughly digested with a few millilitres of aqua regia. The contents of the flask are then heated almost to dryness, care being taken not to char the digest. 20–30 ml. of water are added, together with a few drops of a 1% solution of thallium nitrate. After the formation of the thallium complex is complete, the solution is rendered alkaline with ammonium hydroxide and filtered. The filter-paper is washed thoroughly with ammonia to remove all traces of the salts of extraneous metals.

The residue is then treated with a known volume of 3% hydrochloric acid and filtered. If this solution shows any yellow colour from the nitration of the filter-paper, this colour is measured in a Spekker photo-electric colorimeter and the reading is subtracted from the one obtained later, after the development of the platinum colour. To 10 ml. of the solution or to an aliquot diluted to 10 ml. with the hydrochloric acid is added 0·10 ml. of 40% stannous chloride in concentrated hydrochloric acid. The presence of platinum is shown by the development of a yellow-orange colour, which

* Hunter, D., Milton, R., and Perry, K., *Brit. J. Indust. Med.*, 1945 (ii), 92.

after 15 minutes development time is measured in a Spekker absorptiometer and compared against a graph calibrated with amounts of platinum within the range of 10–500 μg.

Greater sensitivity may be obtained by dissolving the thallous complex in 2·0 ml. of hydrochloric acid and taking readings on a sensitive photo-electric colorimeter with a mirror galvanometer (see p. 246). This micro-reading technique will give results with as little as 0·5 μg. of platinum in solution. It is, however, so delicate that it is difficult to apply in the presence of large amounts of residue from filter-paper digestion.

21. ESTIMATION OF OSMIUM*

PRINCIPLES. Osmium salts will form a coloured complex with thiourea. The salt is said to be $Os[(NH_2CSNH_2)_6]$; if the osmium is in the quadri-valent state, then boiling with stannous chloride will hasten the coloured salt formation. The method is sensitive to about 10 μg. of Os. Ruthenium must be absent, since it forms a blue salt.

REAGENTS.
1. *Thiourea, 10% solution in water.*
2. *Stannous chloride, a 10% solution in 20% v/v hydrochloric acid.*

METHOD. Osmium may be isolated by steam distillation as the tetroxide and a hydrochloric acid solution of the distillate (which should be about $4N$ in acid strength) should contain not less than 10 μg. of Os in 10 ml. 0·5 ml. of thiourea solution and 0·10 ml. of stannous chloride are added to 10 ml. of the osmium solution, and the tube is placed in a boiling water-bath for at least 10 minutes. The colour development is quite rapid if the osmium is in the lower valency state, but proceeds more slowly if it is quadrivalent. After cooling, colour comparison is made against standards.

22. ESTIMATION OF CADMIUM†

PRINCIPLES. Cadmium is separated by repeated precipitation as the sulphide, using a copper salt as the "collector" (p. 259). Then finally it is estimated as colloidal cadmium sulphide.

METHOD. Organic material is destroyed by oxidation with nitric and sulphuric acids in the usual manner (p. 100). The residue is evaporated until the sulphuric acid fumes, taken up in water, and again taken down to the fuming stage. The residue is then diluted to about 10 ml., and 1 ml.

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* Sandell, E., "Colorimetric Methods for Trace Elements" (Interscience Publishers, New York, 1945), p. 347.
† Fairhall, L. T., and Prodan, L., *J. Amer. Chem. Soc.*, 1931, **53**, 1321.

of 0·1% copper sulphate and then 2 g. of sodium citrate are added. The solution is then neutralised with ammonia and adjusted with dilute acid to pH 3·0.

Hydrogen sulphide is passed into the solution for 10 minutes and the solution is allowed to stand for 12 hours to coagulate the precipitate. This is then centrifuged, washed with 0·1% hydrochloric acid, redissolved in 2 ml. of aqua regia, transferred to a basin, and evaporated just to dryness. The residue is dissolved in a little water, 2 g. of citric acid and 1 ml. of 0·1% copper sulphate are added as before, and, after adjustment to pH 3·0, the cadmium sulphide is reprecipitated.

After centrifuging and washing, the precipitate is again dissolved in aqua regia and taken to dryness. The residue is taken up in water, its pH is adjusted to 2·0, copper sulphate is added but not the citrate, and sulphides are again precipitated.

Centrifuging, washing, re-solution of precipitate in aqua regia, and evaporation to dryness are repeated as before. The residue is taken up in 20 ml. of water, 5 drops of 10% potassium cyanide are added to prevent precipitation of copper, followed by 1 ml. of 5% gelatine solution and then 5 ml. of a saturated solution of hydrogen sulphide.

A standard solution of cadmium chloride is treated in the same manner *and at the same time,* and the two solutions compared in the light from a mercury arc; this allows of a tenfold increase in sensitivity.

23. Estimation of Vanadium*

PRINCIPLES. Vanadates with hydrogen peroxide form a reddish-brown pervanadic acid which may be measured colorimetrically. The yellow colour that may be concomitantly produced by titanium may be destroyed by addition of fluorides. The method illustrates the estimation of vanadium in steel.

METHOD. The sample (*ca.* 2 g.) of steel filings is transferred to a beaker and dissolved in about 50 ml. of 50% nitric acid. Any tungsten present will be precipitated as tungstic acid, and this is filtered off. The filtrate is neutralised with dilute ammonia and evaporated to dryness. The residue is taken up in 20 ml. of dilute nitric acid and again filtered from the tungstic acid precipitate. The filtrate is then evaporated to about 10 ml., transferred to a separating funnel, using 10 ml. of 50% hydrochloric acid to assist, and the solution is shaken with 50 ml. of ether, which extracts the bulk of the iron and molybdenum (compare p. 262). The watery layer is removed

* Yoe, J. H., "Photometric Methods of Analysis," Vol. I: "Colorimetry" (Wiley, New York, 1929), p. 391; Wright, E., and Mellon, M., *Ind. Eng. Chem.* (Anal. Edn.), 1937, **9**, 375.

and almost evaporated to dryness, 5 ml. of nitric acid are added, and the solution is again evaporated down to remove all chlorides.

20 ml. of nitric acid are then added and also, after boiling, about 2 g. of potassium chlorate. Manganese and chromium become oxidised, and if the solution is diluted to about 250 ml. a clear solution is obtained.

The hot solution is then neutralised with 50% ammonia and made slightly alkaline. Iron is precipitated with vanadium occluded on the precipitate. This precipitate is filtered off, washed with hot water, and dissolved in 9 ml. of 20% hydrochloric acid. To the solution is then added 1 ml. of hydrofluoric acid to inhibit the effect of iron and titanium. It is then diluted to an appropriate volume and 5 ml. of hydrogen peroxide are added. The red colour which is produced immediately, and is proportional only to the vanadium in solution, is then measured colorimetrically.

24. ESTIMATION OF MOLYBDENUM*

PRINCIPLES. If an acid solution of molybdenum is treated with stannous chloride and then with potassium thiocyanate, an intense reddish-brown colour is produced, and measured accordingly.

METHOD. The sample is appropriately brought into solution and an aliquot, containing about 1 mg. of Mo, is used for analysis. Preliminary separation from other substances is unnecessary, since only tungsten interferes with the colour proportionality. The solution is then made about 5% with respect to hydrochloric acid and 0·8% with respect to stannous chloride. Potassium thiocyanate solution is then added until the concentration is about 1%. The red colour which appears immediately tends to fade for the first 5 minutes and is then stationary for some hours. After standing to reach equilibrium, the colour is extracted by shaking with peroxide-free ether and estimated colorimetrically.

25. ESTIMATION OF TRACES OF AMMONIA BY REACTION WITH PHENOL AND HYPOCHLORITE†

PRINCIPLES. A blue coloration is produced when a weak solution of ammonia is treated with phenol and sodium hypochlorite. One part of ammonia in 100 million may be detected. Nessler's reagent (p. 297) has only one-tenth of this sensitivity.

REAGENTS.
1. *Phenol, 5% solution.*
2. *Sodium hypochlorite, 10% solution.*

* Hurd, L. C., and Allen, H. O., *Ind. Eng. Chem.* (Anal. Edn.), 1935, **7**, 396.
† Cuny, L., *Journ. Pharm. Chem.*, 1926, [8], **3**, 150.

METHOD. 25 ml. of a solution of an ammonium salt (containing about 0·25 mg. per litre) are made just alkaline to phenolphthalein (external indicator) and 20 ml. of 5% phenol are added. To this mixture are then added 20 ml. of 10% sodium hypochlorite, the solution is allowed to stand for 5 minutes and then is diluted with distilled water to 100 ml. It is then heated in a boiling water-bath for 10 minutes, and the colour is compared against a standard solution which has been treated similarly.

26. Estimation of Potassium

Example: Estimation of Potassium in Blood.*

PRINCIPLES. Sodium silver cobaltinitrite produces an extremely insoluble potassium silver salt. The precipitate is decomposed with nitric acid and the cobalt is measured by means of the intense colour given with thiocyanate.

Alternatively, the precipitate is decomposed with caustic soda and the nitrite estimated by the Griess-Ilosvay reagent (see p. 311). This method is sensitive to 0·15 μg. of potassium. Ammonia must be absent.

REAGENTS.

1. *Sodium silver cobaltinitrite.* Dissolve 25 g. of cobalt nitrate in 50 ml. of water and add 12·5 ml. of glacial acetic acid. Dissolve 120 g. of sodium nitrite in 180 ml. of water. Mix 210 ml. of the sodium nitrite solution with the cobalt nitrate solution, and draw air through mixture until nitrous gases are no longer evolved. To 20 ml. of this reagent add 2 ml. of 40% silver nitrate solution; allow to stand over-night and filter before use.

2. *Nitric acid, 20% solution.*

3. *Ammonium thiocyanate, 2% solution in 95% alcohol.*

4. *Sodium tungstate, 10% solution.*

5. *0·66N sulphuric acid.*

METHOD. 2 ml. of serum are transferred to a centrifuge tube and 5 ml. of water and 1 ml. of sodium tungstate solution are added.

After mixing, 1 ml. of 0·66N sulphuric acid is added and the contents of the tube are shaken thoroughly. After a lapse of 15 minutes the tube is centrifuged and 5 ml. of the clear filtrate are transferred to another centrifuge tube.

2 ml. of the cobaltinitrite reagent are then added, drop by drop, with shaking between each addition, observing the precautions given for the analogous volumetric estimation on pp. 168–169. The tube is allowed to stand aside for 2 hours and then is centrifuged. The supernatant fluid

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* Breh, F., and Gaebler, O., *J. Biol. Chem.*, 1930, **87**, 81; Wretlind, K. (*Acta. Physiol. Scandinavia*, 1940, **1–2**, 43), describes a method using 0·01 ml. of blood. Ice and alcohol are used to ensure quantitative precipitation, and the Ilosvay reagent to develop the colour.

is removed by means of a capillary syphon with an upturned tip, and the precipitate is broken up by gentle tapping and then washed three times with 5 ml. portions of distilled water.

The precipitate is then dissolved by gently warming with 1 ml. of 20% nitric acid solution. After cooling, 7 ml. of alcoholic thiocyanate solution are added and the claret coloration is compared against standards in a colorimeter or is measured in a photo-electric absorptiometer.

Alternatively, the precipitate may be dissolved in 5 ml. of $0.2N$ sodium hydroxide, and heated to boiling to coagulate cobalt and silver hydroxides, which are then separated by centrifuging. The supernatant fluid is then made up to 100 ml. with water. 10 ml. of this solution are transferred to a 100 ml. graduated flask and diluted to 70 ml. with water. To this are then added 2 ml. of sulphanilic acid solution (0.5% in 30% acetic acid) and 1 ml. of α-naphthylamine solution (0.5% in 30% acetic acid). The flask is then filled to volume, allowed to stand for 10 minutes, and the colour is compared with standards prepared by the same method.

B. ACID RADICALS

Detailed Procedures

1. COLORIMETRIC ESTIMATION OF FLUORIDE BY INTERACTION WITH A THORIUM LAKE*

PRINCIPLES. Alizarin forms a coloured lake with thorium. In the presence of fluoride a thorium complex is formed which prevents the formation of the lake, and the diminution in colour is therefore a measure of the fluoride content (compare pp. 141 and 191). The method is best carried out after separation of the fluoride by distillation as silicon tetrafluoride (see p. 192), but it may be applied directly to natural waters if the concentrations of interfering ions are below the following values:

(a) Calcium, magnesium, chloride, nitrate, sulphate—100 mg. per 100 ml.

(b) 20 mg. per 100 ml. of silicate do not interfere, but phosphate must be absent, since it produces a turbidity with thorium.

(c) Aluminium and iron, which form lakes with alizarin, must also be absent.

REAGENTS.

1. *Thorium reagent.* Prepare a solution which is $0.001M$ with respect to thorium nitrate and $1M$ with respect to each of the following: sodium sulphate, formic acid, and sodium formate.

2. *Alizarin indicator,* $0.00025M$. Dissolve 0.0855 g. of alizarin mono-sodium sulphonate in 1 l. of water.

3. *Standard fluoride solution.* Dissolve 0.0221 g. of sodium fluoride in 1 l. of water. 1 ml. is equivalent to 0.01 mg. of fluoride.

4. *Nitric acid,* $0.3N$.

METHOD. A series of 100 ml. Nessler tubes containing 2–20 ml. of standard fluoride solution is prepared. 5 ml. of alizarin indicator are added to each and the contents are completed to 100 ml. with water. 5 ml. of thorium reagent are mixed in and the tubes are allowed to stand for 30 minutes.

100 ml. of the water to be examined are transferred to an Erlenmeyer flask, 5 ml. of the alizarin indicator are added, and the sample is titrated with $0.3N$ nitric acid until a pure yellow colour is obtained. The solution is then transferred to a Nessler tube, 5 ml. of the thorium reagent then added, and, after standing for 30 minutes, it is compared against the standard fluoride solutions prepared as above.

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* Talvitie, N., *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15,** 620.

A more accurate end-point may be obtained if standard solutions are measured in a photo-electric instrument and compared with a calibration curve relating fluoride concentration with light extinction.

2. COLORIMETRIC ESTIMATION OF BROMIDE AS TETRABROMOPHENOL-SULPHON-PHTHALEIN*

PRINCIPLES. Bromide is displaced from solution by free chlorine and the resulting free bromine is then combined with phenol red to produce tetra-bromophenol-sulphon-phthalein, the colour of which is measured at a definite pH. Excess of chlorine must be removed with sodium arsenite when the bromination is completed, since otherwise the production of tetrachlorphenol-sulphon-phthalein would occur, thus interfering with colour comparison. The method may be used for estimation of quantities from 1-15 mg. of bromine. In the very lower limits the method is insensitive, whilst quantities of over 15 mg. of bromine will give constant maximum colour.

REAGENTS.

1. *Phenol red.* Grind 10 mg. of phenol red with 1 ml. of N/10 sodium hydroxide and make up to 100 ml. with boiled water.

2. *Borax buffer.* A saturated solution of borax in water at 25° C.

3. *Acetate buffer.* 30·0 ml. of glacial acetic acid and 68 g. of sodium acetate trihydrate per litre of aqueous solution.

4. *Calcium hypochlorite, N/10 solution.*

5. *Sodium nitrite, M/2 solution.*

6. *Sodium arsenite, N/10 solution.*

METHOD. 1·0 ml. of the neutralised bromide solution is transferred to a test-tube and 0·05 ml. of phenol red solution and 0·2 ml. of borax solution are added. After mixing, 0·2 ml. of N/10 calcium hypochlorite is added and the tube is allowed to stand, with occasional shaking, for exactly 4 minutes. At the end of this time, which should not be prolonged beyond 5 minutes, there is added 0·05 ml. of sodium arsenite, followed by 0·2 ml. of acetate buffer to bring the pH to 4·6 or 4·7. The colour thus produced is measured against bromide standards which have been treated in a similar manner.

Reducing agents and ammonia interfere by reacting with the hypochlorite and should be removed. Iodide will react in a similar manner to bromide, but may be removed by adding 2 ml. of N sulphuric acid and 1 ml. of 0·5M sodium nitrite to 10 ml. of solution and boiling gently until the mixture is colourless. The solution should then be tested by adding a further drop of nitrite and if necessary again boiling. Neutralisation must be effected before proceeding, in the manner described above.

* Stenger, V. A., and Kolthoff, I. M., *J. Amer. Chem. Soc.*, 1935, **57**, 831.

3. COLORIMETRIC ESTIMATION OF FREE· CHLORINE OR OF HYPOCHLORITES WITH o-TOLIDINE*

PRINCIPLE. o-Tolidine gives a yellow colour with aqueous solutions containing free chlorine or hypochlorites, and as little as 0·005 parts of chlorine per million can be detected. The method is not specific for chlorine; it is affected by other oxidising agents, e.g. nitrites, and also by certain heavy metals such as iron and manganese. The method can be used up to a limit of 5 parts per million. Palin's method† is more specific.

REAGENT.
o-Tolidine, a 0·1% solution in 10% hydrochloric acid.

METHOD. 100 ml. of water are treated with 2 ml. of o-tolidine solution and allowed to stand for 3 minutes for the colour to develop to its fullest extent. The tint is then compared against standards prepared from hypochlorite diluted with boiled distilled water. Alternatively, the colour may be compared against artificial standards prepared from mixtures of copper sulphate, potassium dichromate and sulphuric acid, or against permanent standards of tinted glass.

4. COLORIMETRIC ESTIMATION OF CYANIDE (AND OF THIOCYANATE) AS CYANOGEN BROMIDE‡

PRINCIPLES. Cyanides react with bromine to form cyanogen bromide. This latter, with pyridine, forms a quarternary salt which in turn reacts with aromatic amines to form coloured di-anil derivatives suitable for colorimetric measurement. Thiocyanates are estimated in the same way, and may be measured in the same solution as cyanides since the latter can be removed as hydrocyanic acid by acidification and aeration. The method is very specific and is sensitive enough to measure 0·3 μg. of cyanide.

REAGENTS.
1. *Bromine water, saturated.*
2. *Sodium arsenite, a* 1·5% *solution in water.*
3. *Pyridine reagent.* 25 ml. of pure redistilled pyridine together with 2 ml. of concentrated hydrochloric acid are made up to 100 ml. with water.
4. *Benzidine hydrochloride, a* 2% *solution in water.*

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* Ellms, J. W., and Hauser, S. J., *Ind. Eng. Chem.*, 1913, **5**, 915.
† Palin (Palin, A. T., *Analyst*, 1945, **70**, 203) has shown that p-aminodimethylaniline may be used to differentiate between *free* chlorine and chloramine, NH_2Cl, in drinking water, since in a buffer at pH 6·8 only chlorine reacts. Chloramine may then be measured by the amount of iodine liberated from potassium iodide. In comparison with the direct o-tolidine method given above, interference by oxidising agents other than chlorine is insignificant.
‡ Aldridge, W. N., *Analyst*, 1944, **69**, 262.

METHOD. To 1 ml. of solution containing up to 3 μg. of cyanide or 6 μg. of thiocyanate is added 0·5 ml. of saturated bromine water and then 0·5 ml. of sodium arsenite solution, which destroys any excess of free bromine. To this solution are added 5 ml. of pyridine reagent and then 0·2 ml. of benzidine solution. The red colour, which is fully developed in 10 minutes, is apparently constant for a further 10 minutes if measured in a photo-electric instrument using an Ilford 2303 blue filter. Visually there can be noticed a perceptible colour change from orange to red, so that if a colorimeter is used then standards must be prepared at the same time as the sample which is being analysed.

5. COLORIMETRIC ESTIMATION OF NITRATE WITH PHENOL DISULPHONIC ACID*

PRINCIPLES. Phenol disulphonic acid reacts with nitrates to form highly coloured derivatives. Chlorides interfere, but the method as given below can be regarded as accurate if the concentration of chlorine does not exceed 0·1%. 5 μg. of nitrate may be measured by this method.

REAGENT.
Phenol disulphonic acid reagent. Mix 4 g. phenol with 4 ml. of water and add 100 ml. of concentrated sulphuric acid. After heating for 6 hours at 80° C., cool the solution and dilute to 500 ml. with water. This stock solution is diluted with an equal volume of an acid made by diluting 300 ml. of sulphuric acid to 500 ml. with water.

METHOD. The sample of water containing not more than 0·1 mg. of nitrogen as nitrate is diluted to 25 ml. with distilled water and transferred to an evaporating dish of about 3½ in. diameter. 2 ml. of the phenol disulphonic acid reagent are added and the mixture is stirred and evaporated over a boiling water-bath until water is no longer expelled. Care must be taken during the evaporating process to avoid the presence of dried spots on the sides of the dish. This may be prevented by tilting the liquid frequently round the sides of the container.

When water ceases to be volatilised, the sides of the dish are washed down with a jet of water and evaporation is again carried out until water is no longer evolved. This whole process is repeated once more, and then the residue is transferred quantitatively, with water, to a 100 ml. Nessler cylinder. The solution is made alkaline by addition of 3 ml. of strong ammonia, diluted to 100 ml. with water, and the yellow colour which is produced is compared against standard solutions of potassium nitrate which have been treated in a similar manner.

Other useful methods include (i) the use of 2 : 4-xylen-1-ol, the nitro derivative of which is volatile in steam and may thus be removed from

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* Frederick, R. C., *Analyst*, 1919, **44**, 281.

interfering material,* and (ii) the reduction of nitrate to nitrite and subsequent estimation of nitrite as described below (compare Bray, R., *Soil Science*, 1945, **60**, 219).

6. COLORIMETRIC ESTIMATION OF NITRITE BY FORMATION OF AN AZO DYE[†]

PRINCIPLES. The Griess-Ilosvay test for nitrous acid depends upon the diazotisation of sulphanilic acid and the coupling of the product with alpha naphthylamine to produce a red azo dye.

The method has been modified by Shin, who diazotised sulphanilamide and coupled the resulting compound with N-(1-naphthyl)-ethylenediamine dihydrochloride. This improved method gives a much brighter colour, which reaches its maximum intensity more rapidly and remains stable for some hours. Also, the use of an unstable nitrite standard solution may be avoided, since sulphanilamide itself may be used in its place.

REAGENTS.

1. *Hydrochloric acid, 5N.*

2. *p-Aminobenzene sulphonamide, 0·2% solution.*

3. *Ammonium sulphamate, 0·5% solution.*

4. *N-(1-naphthyl)-ethylenediamine hydrochloride, 0·1% solution.*

METHOD. The neutral solution of nitrite (not more than 0·05 mg.) is contained in about 35 ml. of water. 1·0 ml. of 0·5N hydrochloric acid is added, followed by 5 ml. of the 0·2% solution of sulphanilamide. After standing for 3 minutes 1 ml. of 0·5% ammonium sulphamate is added, and after a further 2 minutes 1 ml. of 0·1% N-(1-naphthyl)-ethylenediamine hydrochloride solution. The mixture is diluted to 50 ml. with water and allowed to stand for 3 minutes for full development of the red colour.

7. COLORIMETRIC ESTIMATION OF SULPHIDE AS METHYLENE BLUE[‡]

PRINCIPLES. Hydrogen sulphide reacts with p-amino dimethylaniline in the presence of an oxidising agent to form methylene blue.

REAGENTS.

1. *p-Amino dimethylaniline hydrochloride, 0·04% in 5N hydrochloric acid,* freshly prepared.

2. *0·1M acid solution of ferric chloride.* 27 g. of $FeCl_3 6H_2O$ are dissolved in 500 ml. concentrated hydrochloric acid and diluted to 1 l. with water. Dilute five times just prior to use.

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* Blom, J., and Treschow, C., *Z. Pflanz. Düngung. Bodenk*, 1929, **13a**, 159. Cf. Allport, "Colorimetric Analysis" (London, 1945), p. 145.

† Shin, M. B., *Ind. Eng. Chem.* (Anal. Edn.), 1941, **13**, 33.

‡ Almy, L., *J. Amer. Chem. Soc.*, 1925, **47**, 1381.

3. *Zinc acetate*, 20% *solution*. 135 g. of glacial acetic acid are treated with an excess of zinc oxide, diluted to 1 l. with water and filtered. This solution is diluted to 2% as required.

METHOD. The sample to be analysed is introduced into a tall cylinder marked *a* (see fig. IV.15) and 50 ml. of water are added. The cylinder is fitted with a three-holed stopper, through one hole of which is placed a dropping funnel *b*; through a second is introduced an inlet tube *e* passing down to the bottom of the cylinder. The third hole contains a bent tube which passes to the bottom of a 100 ml. distilling flask *c*, a side-arm of which is extended and bent to pass to the bottom of a 100 ml. volumetric flask *d*. 50 ml. of 5N hydrochloric acid are introduced into the dropping funnel *b* and 30 ml. of 0·6% zinc acetate solution are placed in the distilling flask *c*. 20 ml. of zinc acetate are also placed in the volumetric flask.

Carbon dioxide is passed through the apparatus *via* the inlet tube *e* at a pressure equivalent to 400 mm. of water. The acid is allowed to flow slowly into the cylinder *via* the dropping funnel and the gas flow is continued for 15 minutes. The solution in the distilling flask is then transferred to the volumetric flask, using 40 ml. of water to assist. To this solution are added 5 ml. of the *p*-aminodimethyaniline reagent and 1 ml. of 0·02M ferric chloride. After mixing, the flask is set aside for 2 hours, completed to 100 ml. with water, and the

Fig. IV.15. Separation of Hydrogen Sulphide.

blue colour is compared in the colorimeter against a standard sulphide solution which has been developed under the same conditions and at the same time.

For comparison hydrogen sulphide, prepared from ferrous sulphide and hydrochloric acid, is scrubbed with cool water and passed slowly for about 2 minutes into 300 ml. of cool boiled distilled water.

An aliquot portion of the solution is estimated by adding excess of 0·01N iodine and back-titrating with 0·01N sodium thiosulphate solution. An amount equal to 0·01 mg. of hydrogen sulphide is then added to 90 ml. of 0·6% zinc acetate solution and the colour is developed as above. If stronger solutions of sulphide, of up to 0·6 mg. of hydrogen sulphide in the

zinc acetate solution, have to be analysed, then colorimetric comparison should be made against a standard of closely similar concentration.

8. COLORIMETRIC ESTIMATION OF SULPHATE AS DIAZOTISED BENZIDINE*

Example: Colorimetric Estimation of Sulphate in Blood.

PRINCIPLES. Benzidine sulphate is insoluble in acetone. The benzidine in the precipitate is diazotised, coupled with thymol, and colorimetrically estimated. The method is suitable for a range from 0·0025 up to 0·08 mg. sulphate, calculated as sulphur.

REAGENTS.

1. *Trichloracetic acid,* 20% *solution.*
2. *Benzidine,* 0·5% *solution in acetone.*
3. *Hydrochloric acid N.*
4. *Sodium nitrite,* 0·1% *solution.*
5. *Sodium hydroxide,* 15% *solution.*
6. *Thymol,* 1% *solution in* 10% *sodium hydroxide solution.*

METHOD. Into a centrifuge tube are placed 2·0 ml. of 20% trichloracetic acid, 6·0 ml. of water, and 2·0 ml. of serum. The mixture is shaken and centrifuged. 2·5 ml. of the supernatant fluid are transferred to another centrifuge tube, to which are added 5 ml. of the solution of benzidine in acetone. The contents of the tube are mixed and allowed to stand for 30 minutes to complete precipitation. The tube is centrifuged, supernatant fluid is removed, the precipitate is twice washed with acetone, and finally the tube is inverted over filter-paper to dry. The precipitate is then dissolved with warming with 1 ml. of hydrochloric acid. After cooling, 0·5 ml. of freshly prepared 0·1% sodium nitrite solution is added, and exactly 1 minute later 2·5 ml. of 15% sodium hydroxide, followed by 2·5 ml. of 1% thymol in alkali. Before measurement a period of 15 minutes is allowed to elapse in order for the colour to reach its maximum intensity.

9. COLORIMETRIC ESTIMATION OF SILICATE AS MOLYBDENUM BLUE†

Example: Estimation of Silica in Organic Matter.

PRINCIPLES. Silicates form with molybdic acid a complex which is readily reducible to molybdenum blue. Phosphate also reacts in this manner (p. 314), but may be prevented from interfering by addition of oxalic or citric acids. In this method the bulk of the phosphate is removed as

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* Cuthbertson, D., and Tomsett, S., *Biochem. J.*, 1931, **25**, 1237.
† King, E. J., *Bull. Soc. Chim. Biol.*, 1930, **12**, 903; Amor, A. J., "Chemical Aspects of Silicosis" (Lewis, London, 1942).

magnesium ammonium phosphate in the presence of acetic acid, when the traces which remain do not interfere. If comparison against standards is to be accurate, it is essential that the concentrations of reagents be rigidly adhered to.

REAGENTS.

1. *Boric acid, saturated solution.*

2. *Magnesium nitrate, 10% solution.*

3. *Sodium hydroxide solution, silica-free.* Dissolve 2·3 g. of metallic sodium in water in a platinum crucible and dilute to 100 ml.

4. *Ammonium chloride, N solution.*

5. *Acetic acid, 10%.*

6. *Ammonium molybdate, 10%.*

7. *Hydroquinone, 0·5% in 20% sodium sulphite solution.*

METHOD. The sample is transferred to a platinum crucible, dried, and ashed. The residue (*ca.* 0·5 g.) is treated with 1 ml. of boric acid solution, 1 ml. of magnesium nitrate solution, and 3 ml. of concentrated nitric acid. The mixture is heated on a boiling water-bath until solution results, and is then taken to dryness on an electric hotplate. The residue is then heated strongly over a Bunsen flame until a white ash is obtained. The ash is dissolved in a few drops of nitric acid, evaporated to dryness, and the residue is taken up in 10% acetic acid solution.

2·5 ml. of silica-free sodium hydroxide solution are added, and the mixture is warmed on a water-bath until the silica has dissolved.

5 ml. of *N* ammonium chloride are then added, and the mixture is set aside for about ½ hour to allow of precipitation of magnesium ammonium phosphate, which is removed by filtration. The filtrate is neutralised with 10% acetic acid solution, 3 ml. of which are added in excess. To this solution are then added 3 ml. of 10% ammonium molybdate and 2 ml. of hydroquinone-sodium sulphite solution, and the volume is made up to 25 ml. with water. After standing at room temperature for 30 minutes the blue colour is compared against standard solutions of silica which have been treated similarly.

10. COLORIMETRIC ESTIMATION OF PHOSPHATE AS MOLYBDENUM BLUE*

PRINCIPLES. The best method for the estimation of phosphates is that involving reduction of the complex formed when molybdic acid is mixed with a phosphate. Stannous chloride is the most satisfactory reducing

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* Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, 1927, **75**, 517; Milton, R., and Obermer, E., *J. Lab. and Clin. Med.*, 1932, **17**, 792. A much more sensitive method involves the use of quinaldine red together with the molybdate reagent. It is, however, less specific (see Soyenoff, *J. Biol. Chem.*, 1947, **168**, 447).

agent of the many that have been suggested. If the acid concentration is rigidly maintained, then no interference by silicates is encountered; and if the concentration of stannous chloride is kept within defined limits, then no auto-reduction of molybdic acid occurs. Arsenate reduces in the same manner as phosphate, but does not give a colour if reduced to arsenite by boiling with sulphurous acid prior to the addition of molybdic acid. The colour is constant for at least 24 hours.

REAGENTS.

1. *Sulphuric acid*, 10N. Add 37·5 ml. of concentrated sulphuric acid to 100 ml. of water.

2. *Sodium molybdate*, 7·5%. Molybdic acid precipitated from solution by acid treatment is washed and redissolved in the appropriate amount of sodium hydroxide.

3. *Stannous chloride solution*. Dissolve 10 g. of stannous chloride in 25 ml. of hydrochloric acid. Dilute 1 ml. to 200 ml. with water just prior to use to give a 0·20% solution.

METHOD. The sample is diluted so that the phosphate concentration is not greater than 0·05 mg. in 5 ml. To this volume are added exactly 2·0 ml. of water, 1·0 ml. of 10N sulphuric acid, 1·0 ml. of sodium molybdate, and, after mixing, 1·0 ml. of the diluted (0·20%) stannous chloride. If the quantities have been measured accurately, the colour (which is fully developed in a few minutes and which is stable for many hours) is directly proportional to the concentration of phosphate. The colour may be compared directly against similarly treated standards, and is admirably suited for measurement in a photo-electric colorimeter, since the depth of colour is always reproducible irrespective of the temperature.

11. COLORIMETRIC ESTIMATION OF BORATE WITH TURMERIC*

PRINCIPLES. Boric acid is isolated as methyl borate. After hydrolysis with alkali and acidification the product is evaporated down to dryness with acid turmeric (curcumin), and produces an alcohol-soluble red colour suitable for colorimetric comparison. Care must be taken to free from soluble boron compounds all apparatus used.

REAGENTS.

1. *Turmeric-coated sand.* 20 g. of powdered turmeric is extracted with ether in a Soxhlet apparatus and the ethereal solution is diluted to 250 ml. with ether. 100 ml. of this is mixed with 1 kg. of acid-washed silver sand and evaporated to dryness on a water-bath.

2. *Oxalic acid*, 4·5% solution in water.

* Robinson, K., *Analyst*, 1939, **64**, 324.

3. *Barium hydroxide, saturated.*

4. *Syrupy phosphoric acid, sp. gr.* 1·75.

5. *Methyl alcohol.*

6. *Sodium hydroxide, N.*

METHOD. The sample, containing about 0·05 mg. of B_2O_3, is made alkaline with barium hydroxide, dried, and ignited. The residue is transferred to a distilling flask and is washed in with 15 ml. of methyl alcohol and 5 ml. of syrupy phosphoric acid. The flask is immersed in a boiling water-bath and the alcohol is distilled into a receiver containing 0·5 ml. of N sodium hydroxide. A flask containing 25 ml. of methyl alcohol is then placed in front of the distilling flask and, with the latter maintained at the boiling-point, alcohol vapour is passed through the borate solution. In this way all the borate is removed as methyl borate. This alkaline distillate is evaporated to dryness in a porcelain dish. The residue is then dissolved in 4·5 ml. of oxalic acid solution and 40 g. of turmeric-coated sand are added. The mixture is well stirred and then is evaporated to dryness. 2 ml. of oxalic acid are again added, and after a thorough mixing, evaporation to dryness is again carried out. The sand is then extracted thoroughly with 10 ml. lots of 70% alcohol until it is colourless. The volume of extract is made up to 100 ml. and the tint is measured in a colorimeter or photo-electric absorptiometer. In each case a yellow screen must be fitted to avoid error due to the yellow colour of the turmeric.

C. ORGANIC COMPOUNDS

1. ALCOHOLS

Methods (*a*) and (*b*) illustrate procedures for the lower alcohols.

Method (*c*) can be used for many higher alcohols or their esters.

(a) The Estimation of Methyl Alcohol with Phenyl-Hydrazine after Conversion to Formaldehyde*

PRINCIPLES. Methyl alcohol is oxidised with potassium permanganate to formaldehyde; the latter reacts with phenyl-hydrazine in the presence of potassium ferricyanide to produce a violet coloration.

Other volatile alcohols, aldehydes, or ketones must not be present in the sample for analysis.

Quantities of the order of 0·1 mg. can be assessed.

The original method used ammonium persulphate as an oxidising agent, but permanganate† is preferred, since then over-oxidation is more easily avoided.

REAGENTS.

1. *Potassium permanganate.* 3 g. in 85 ml. of distilled water and 15 ml. 85% phosphoric acid.

2. *Potassium ferricyanide, 2·5% solution, freshly made.*

3. *Phenyl-hydrazine hydrochloride, 1% solution, freshly made and filtered.*

4. *Oxalic acid, 5% solution.*

METHOD. A watery sample containing the methyl alcohol is transferred to a distilling flask and distilled until 60% of the volume has come over. This process is repeated until the methyl alcohol is contained in 10 ml. of solution. To 2 ml. of solution is added 1 ml. of acid potassium permanganate reagent. After standing for 10 minutes excess permanganate is removed by the addition of 1 ml. of oxalic acid solution. The volume is then made up to 10 ml. with water. To this are then added 2 ml. of phenyl-hydrazine hydrochloride solution and, after mixing well, 2 ml. of potassium ferricyanide solution. The mixture is then made strongly acid by the addition of 3 ml. of concentrated hydrochloric acid, and after a short time interval the resulting violet colour is compared against standard solutions of methyl alcohol which have been similarly treated.

* Schryver, S. B., and Wood, C. C., *Analyst*, 1920, **45**, 165.
† Wright, L. O., *Ind. Eng. Chem.*, 1927, **19**, 750.

(b) The Estimation of Ethyl Alcohol or of Methyl Alcohol using Schiff's Reagent*

PRINCIPLES. Ethyl alcohol is oxidised in aqueous solution with potassium dichromate to acetaldehyde.   The acetaldehyde is distilled and the distillate treated with Schiff's reagent, which will produce a colour for measurement purposes.

Methyl alcohol may be similarly estimated after oxidation to formaldehyde.†

Other alcohols or aldehydes should not be present in the mixture for analysis.

The method, which will indicate ethyl alcohol of the order of 0·1 part per million, will give satisfactory results for total concentrations up to 5 parts per million.

For the estimation of ethyl alcohol in blood see p. 402.

REAGENTS.

1. *Potassium dichromate, saturated solution.*

2. *Schiff's reagent.* 0·2 g. of rosaniline is dissolved in 120 ml. of hot water, and after cooling 20 ml. of 10% sodium bisulphite solution are added. The mixture is set aside for 10 minutes, treated with 2 ml. of hydrochloric acid, and finally diluted to 200 ml. with distilled water.   This solution should be allowed to stand for some hours before use, when it will become nearly colourless.   If kept stoppered no deterioration occurs.

METHOD. 20 ml. of the sample are transferred to a 100 ml. "Quick-fit" distillation flask, 5 ml. of potassium dichromate solution are added, and the flask is connected to a condenser with a spray trap interposed. 1 ml. of concentrated sulphuric acid is then added and the contents of the flask are slowly brought to the boil. Oxidation to acetaldehyde is quite rapid, and the whole of the aldehyde passes over with the first 5 ml. of distillate.   The distillate is made up to a known volume (e.g. 10 ml.) and to 5 ml. is added 0·5 ml. of Schiff's reagent.   Colour development takes about 1 hour to reach a peak at room temperature, but may thereafter be read at any time up to 8 hours.

The method is standardised by submitting known amounts of ethyl cohol to the full procedure described above.

(c) Estimation of Amyl Alcohol‡

PRINCIPLES. When amyl alcohol in ethyl alcoholic solution is treated with furfural and sulphuric acid, a rose-red colour is produced.   It is probable that under the action of sulphuric acid, amyl alcohol is dehydrated to

* Argenson, M. G., *Bull. Soc. Chim.*, 1902, **27**, 1000.
† Alyea, H. N., and Bäckström, H. L. J., *J. Amer. Chem. Soc.*, 1929, **51**, 90.
‡ Komarowsky, A., *Chem. Ztg.*, 1903, **27**, 807; Bassett, H., *Ind. Eng. Chem.*, 1910, **2**, 389.

amylene, which gives a colour with aldehydes. Many esters of higher alcohols react in a similar manner. The method is of value for the detection of fusel oil in spirits, etc., and will readily estimate 0·001% of amyl alcohol in ethyl alcohol.

REAGENTS.

1. *Furfural, 1% solution in ethyl alcohol.*
2. *m-Phenylene-diamine hydrochloride, solid.*

METHOD. About 25 ml. of the alcoholic solution to be tested are treated with 5 ml. of *N* sodium hydroxide solution. The mixture is heated under a reflux for 1 hour to saponify esters; 25 ml. are then distilled over and cooled. The distillate is then treated with 2 g. of *m*-phenylene-diamine hydrochloride and refluxed for 1 hour to destroy any aldehydes. The solution is again distilled, and to 1·0 ml. of this distillate is added 0·1 ml. of furfural solution. 1·5 ml. of concentrated sulphuric acid is carefully poured down the side of the tube, and, after mixing, the tube is cooled under running water.

The sample is then heated in a boiling water-bath for 3 minutes, cooled, and the resultant colour is then measured optically. Comparison may be made in a colorimeter against a standard solution of amyl alcohol which has been treated with furfural and sulphuric acid in a similar manner. Alternatively, the colour may be measured in a photo-electric instrument and assessed against a calibration curve.

(d) Estimation of Glycerol with Codeine*

PRINCIPLES. Glycerol is oxidised with bromine to dihydroxyacetone. The latter is changed to methylglyoxal by dehydration, and coupled with codeine to produce a reddish colour.

Fats and soaps must previously be removed by adsorption on to charcoal.

The method is suitable for the estimation of glycerol in mixtures and will detect quantities of the order of 0·1 mg.

REAGENTS.

1. *Decolorising charcoal.*
2. *Sodium hydroxide, 2·5% solution.*
3. *Sulphuric acid, 2N.*
4. *Bromine water, saturated.*
5. *Codeine solution, 10% in alcohol.*

METHOD. The sample is shaken with decolorising charcoal, stood for 15 minutes, and twice filtered through the same paper. To 1 ml. of the filtrate are added 1 ml. of caustic soda solution, 1 g. of kaolin, and 10 ml. of alcohol. The mixture is shaken and filtered. After heating 6 ml. of this filtrate for 1 hour on a boiling water-bath, 1·5 ml. of dilute sulphuric

* de Coquet, C., *Bull. Soc. Pharm. Bordeaux*, 1928, **66**, 235.

acid and then 2 ml. of saturated bromine water are added.   The contents of
the tube are then diluted to approximately 10 ml., heated on a water-bath
for 20 minutes, and then finally boiled until all the bromine has been removed.
After cooling, the contents of the tube are diluted to 20 ml.   2·0 ml. of this
solution are then treated with 2·0 ml. of alcoholic codeine solution, and
5·0 ml. of concentrated sulphuric acid are carefully added while the tube is
held under cold water to prevent overheating.   After mixing, the tube is
stood aside for 10 minutes, and then heated to boiling for exactly 5 minutes.
The contents of the tube are again cooled by immersion in cold water, and,
after a further interval of 10 minutes, the resulting colour is measured in
the usual manner by comparison against standard solutions of glycerol.

<h2 style="text-align:center">2. ALDEHYDES AND KETONES</h2>

Methods (a), (b), and (g) are specific reactions; methods (c) to (f) are general
procedures for aldehydes or ketones closely analogous to those mentioned
in the text.

(a) The Estimation of Acetone with o-nitro-benzaldehyde*

PRINCIPLES. If a solution of acetone is treated with o-nitro-benzaldehyde
in the presence of caustic soda, condensation occurs with the production of
indigo, which may be measured colorimetrically.

The reaction is specific for acetone.   Quantities of the order of 1 mg.
can be detected.

REAGENTS.

1. *o-Nitro-benzaldehyde, a freshly prepared* 50% *solution in acetone-free
ethyl alcohol.*

2. *Sodium hydroxide,* 30% *aqueous solution.*

METHOD. Acetone is removed from the sample to be tested by direct
distillation.   10 ml. of the distillate, which should not contain more than
20 mg. of acetone, are treated with 1 ml. of freshly prepared o-nitro-benzal-
dehyde solution.   After thorough mixing, 0·5 ml. of caustic soda is added
and the pigment is allowed to develop in the dark for 15 minutes.   The
indigo blue is then measured colorimetrically.   If a photo-electric method
is adopted, the instrument is calibrated by taking a series of tubes containing
0–20 mg. acetone in 10 ml. of water, adding o-nitro-benzaldehyde and
caustic soda to each, and allowing the colour to develop in the manner
described above, before making optical measurements.   When dealing with
low concentrations the use of a yellow screen in the eyepiece of the colorimeter
is advisable.

* Nicholls, J. R., and Adams, C. A., *Analyst,* 1929, **54**, 2.

(b) The Estimation of Acetone by Salicylic Aldehyde*

PRINCIPLES. In alkaline solution acetone reacts with salicyl-aldehyde to form dihydroxydibenzylidene acetone, which is measured colorimetrically. Quantities of the order of 0·1 mg. can be estimated.

REAGENTS.

1. *Sulphuric acid,* 50%.

2. *Sodium hydroxide,* 40%.

3. *Salicyl-aldehyde, a freshly made* 20% *solution in* 95% *alcohol.*

METHOD. The sample, which should contain about 0·1 mg. of acetone, is transferred to a 100 ml. distillation flask, and diluted to 75 ml. with water. After acidification with 0·5 ml. sulphuric acid, the flask is connected to a condenser with all-glass joints. The acetone is distilled over with the first 25 ml. of liquid, which is made up to a given volume.

5·0 ml. of this sample are transferred to a test-tube, 4·0 ml. of caustic soda solution are added, and then, after mixing, 1·0 ml. of salicyl-aldehyde solution. The tube is warmed to 50° C. for 20 minutes and then stood at room temperature for a further ½ hour in order that the peak of colour development can be reached. Comparison may be made against standard solutions of acetone which have been treated in a similar manner.

(c) The Estimation of Citral with *Meta*-phenylene-diamine†

PRINCIPLES. When many aldehydes of the terpene group are treated with *m*-phenylene-diamine, a yellow colour is produced. Quantities of the order of 1 mg. may be estimated.

REAGENT.

m-Phenylene-diamine hydrochloride. Dissolve 1 g. of the solid substance in about 50 ml. of 80% alcohol. Dissolve 1 g. of oxalic acid in a further 50 ml. of 80% alcohol. Mix the two solutions and dilute to 100 ml. Add 5 g. of fuller's earth, shake well, allow to settle, and then filter twice through the same filter-paper to obtain a perfectly clear and colourless solution. This reagent should be freshly prepared.

METHOD. A known quantity of the citral-containing substance, e.g. 0·5 g. of lemon oil, is dissolved in 50 ml. of 95% alcohol. To 5 ml. of this dilution are added 10 ml. of the *m*-phenylene-diamine reagent, and the mixture is diluted to 50 volumes with 95% alcohol.

The yellow colour is then compared against standard solutions of pure citral which have been treated similarly.

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* Csonka, F. A., *J. Biol. Chem.,* 1916, **27**, 209.
† *U.S. Dept. Agric. Bur. Chem. Bull.,* 1910, **137**, 70.

(d) The Estimation of Acetaldehyde with Hydroquinone*

PRINCIPLES. Hydroquinone produces with acetaldehyde an orange-brown colour, sensitive to 1 mg. of the latter. Certain other aldehydes react similarly. The method was elaborated for the estimation of lactic acid in blood—the lactic acid is converted to acetaldehyde by sulphuric acid dehydration.

REAGENTS.

1. *Hydroquinone, 20% solution in alcohol.*

2. *Copper sulphate, 10% solution.*

METHOD. The aldehyde, which should be contained in 1 ml. of water, is treated with 0·1 ml. of copper sulphate solution followed by 4 ml. of concentrated sulphuric acid. 0·1 ml. of hydroquinone solution is then added and, after mixing, the tube is heated in a boiling water-bath for 15 minutes. After cooling, the yellow colour is compared against that given by standard solutions of acetaldehyde which have been treated similarly.

(e) The Estimation of Acrylic Aldehyde with Benzidine Acetate†

PRINCIPLES. When benzidine acetate is heated with acrylic aldehyde (or any other aldehyde), a yellow colour is produced which is proportional to the concentration of the latter.

REAGENT.

Benzidine acetate. Dissolve 1 g. of recrystallised benzidine in 100 ml. of glacial acetic acid.

METHOD. The sample, which should contain less than 0·1 mg. of the aldehydes, is contained in 10·0 ml. of water. To this is added 1·0 ml. of freshly prepared benzidine acetate solution, and the contents of the tube are brought to boiling and kept at 100° C. for 1 minute. After cooling for 15 minutes the yellow colour is compared against standard solutions of the required pure aldehyde which have been treated in a similar manner.

(f) The Estimation of Furfural with Aniline‡

PRINCIPLES. When a solution of furfural is allowed to react with an aniline salt a red colour is produced.

The same method is used for the estimation of pentoses, e.g. for the detection of wood fibre in paper, etc. (see p. 337).

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* Dische, Z., and Laszlo, D., *Biochem. Z.*, 1927, **187**, 344.

† Zhitkova, A., Kaplun, S., and Ficklen, J., "Estimation of Poison Gases and Vapours in Air," 1936, p. 134. Cf. Snell, "Colorimetric Methods of Analysis" (Chapman and Hall, London, 1937), Vol. II, p. 68.

‡ Stillings, R. S., and Browning, B. L., *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 499.

REAGENTS.

1. *Sodium hydroxide*, 10% *solution.*

2. *Aniline acetate.* Dissolve 5 g. of colourless, freshly distilled aniline in 50 ml. of glacial acetic acid.

METHOD. The sample containing furfural is distilled into a 100 ml. graduated flask. The quantity of furfural should be of the order of 0·2 mg. The solution is neutralised to phenolphthalein with dilute caustic soda and diluted to approximately 40 ml. 2 g. of solid sodium chloride and, after mixing, the total bulk of the aniline acetate solution prepared as above are added. The mixture is then rapidly brought to 20° C., diluted to 100 ml. with water, and maintained at this temperature for 55 minutes in the absence of light. The intensity of the colour is then compared against that produced by standards which have been treated similarly.

(g) The Estimation of Epihydrinaldehyde as a Measure of Rancidity in Fats*

PRINCIPLES. It was shown by Kreis that if an oxidised fat is treated with an ethereal solution of phloroglucinol in the presence of concentrated hydrochloric acid, a red colour is produced which indicates the degree of rancidity in the fat. The compound responsible for this colour is said to be epihydrinaldehyde, $\underset{CH_2-CH-CHO}{\overset{O}{\diagup\diagdown}}$, which may be produced in aqueous solution by oxidation of acrolein with hydrogen peroxide.

Pyke's modification of this method is given below.

REAGENT.

Phloroglucinol, 1% *solution in acetone.*

METHOD. 1·0 g. of fat is dissolved in 10 ml. of the acetone solution of phloroglucinol, 0·5 ml. of concentrated sulphuric acid is added, and the mixture is maintained at 10° C. for 15 minutes. The resulting red colour is then measured.

The original method recommends the use of the Lovibond tintometer and expresses the results in Lovibond red units. The method may also be adapted for use with the Pulfrich photometer or any other such measuring instrument, and the result given in optical densities. It is essential that water should be absent, since even traces inhibit the colour reaction. Also the temperature must be very carefully controlled in order to obtain consistent results.

3. ACIDS AND THEIR DERIVATIVES (ESTERS, AMIDES, ETC.)

The following examples illustrate some specific colour reactions for important organic acids.

* Pyke, M. A., *Analyst*, 1935, **60**, 515.

(a) Estimation of Formic Acid*

PRINCIPLES. The precipitate resulting from the reaction between formic acid and mercuric chloride is dissolved in phospho-molybdo-tungstic acid and the blue colour which develops on addition of alkali is measured.

Formaldehyde, ascorbic acid, and other reducing substances which may react with mercuric chloride must be absent.

REAGENTS.

1. *Mercuric chloride reagent.* 20 g. of mercuric chloride, 30 g. of sodium acetate, and 8 g. of sodium chloride are dissolved in 100 ml. of water.

2. *Diatomaceous earth.* 20 g. of fuller's earth are suspended in 100 ml. of water.

3. *Phospho-molybdo-tungstic acid reagent.* 10 g. of phosphotungstic and 130 g. of phosphomolybdic acids are dissolved in water to give a volume of 400 ml.; 50 ml. of concentrated hydrochloric acid and 50 ml. of 85% phosphoric acid are then added and the whole is mixed.

4. *Sodium carbonate*, a saturated aqueous solution.

METHOD. 1 ml. of the solution (containing up to 30 μg. of formic acid) is transferred to a centrifuge tube and 0·5 ml. of mercuric chloride solution is added. The mixture is heated on a steam-bath for 3 hours, care being taken to avoid undue evaporation. It is then cooled and 2 ml. of the diatomaceous earth suspension are added to assist in taking down the precipitate. This precipitate is separated by centrifuging, washed twice with 15 ml. of water and then is broken up, treated with 1 ml. of the phospho-molybdo-tungstic acid reagent and heated on a steam-bath for 15 minutes.

Immediately after centrifuging down the diatomaceous earth, 4 ml. of saturated sodium carbonate solution are added and the blue colour is then measured photo-electrically.

(b) Estimation of Lactic Acid (in Blood)†

PRINCIPLES. The blood is deproteinised and the filtrate is treated with copper sulphate and alkali to remove reducing substances (compare p. 210). Under the action of sulphuric acid, lactic acid is then converted to an aldehydic substance which reacts with veratrol to give a magenta colour.

REAGENTS.

1. *Zinc sulphate, 10% solution.*

2. *Sodium hydroxide, N/2 solution.*

3. *Copper sulphate, 15% solution.*

4. *Fine powdered lime, freshly calcined.*

5. *Veratrol, 0·125% solution in absolute alcohol.*

* Morton Grant, W., *Analyt. Chem.*, 1947, **19**, 206.
† Milton, R., *Analyst*, 1936, **61**, 91.

METHOD. Blood is collected in a tube containing 1 mg. of ammonium fluoride for every millilitre of blood taken. 1·0 ml. of the blood is pipetted into a centrifuge tube and mixed with 3·0 ml. of water. 1·0 ml. of zinc sulphate solution is added, followed by 1·0 ml. of sodium hydroxide solution added drop by drop with shaking. The mixture is then thoroughly shaken, allowed to stand for a few minutes, and centrifuged at high speed for ¼ hour. 3·0 ml. of the clear liquid are then pipetted into another centrifuge tube which has a graduation at 5 ml. To this is added 1·0 ml. of copper sulphate solution, followed by 1 g. of lime. The contents of the tube are mixed by careful inversion, and then the volume is made up to 5·0 ml. with water. The tube is shaken thoroughly and allowed to stand for ½ hour, and then it is centrifuged. The supernatant fluid is poured into a filter-stick packed with a layer of acid-washed asbestos. The first few drops of liquid that pass through the filter are discarded. The remainder of the filtrate is collected in a test-tube. 1·0 ml. of the filtrate, which represents 0·1 ml. of blood, is pipetted into a clean, dry test-tube, and 3·0 ml. of concentrated sulphuric acid are introduced down the side of the tube. The contents are carefully mixed by tilting the tube and rotating it gently. The tube is placed in a boiling water-bath for 5 minutes and then cooled to 20° C. 0·15 ml. of the veratrol solution is added, and, after mixing, the tube is placed for 20 minutes in a beaker of water maintained at 20° C. The depth of the magenta colour is then compared against a suitably prepared standard or measured in a photo-electric colorimeter. If the latter method is used, it is necessary to calibrate the instrument against standard solutions of lactic acid as follows:

171 mg. of pure crystallised calcium lactate are dissolved in 100 ml. of water. This solution will contain 1 mg. of lactic acid per millilitre. 5 ml. of this solution are diluted to 100 ml. with water, and from this is made a series of dilutions containing from 0·005–0·03 mg. of lactic acid per millilitre. 1 ml. of each dilution is then treated exactly as in the above technique from the stage following the copper+lime treatment. The colours which develop are subsequently measured on the instrument, and a graph is plotted to relate instrument readings to concentration of lactic acid.

(c) Estimation of Pyruvic Acid (in Blood)*

PRINCIPLES. Pyruvic and other ketonic acids produce insoluble derivatives with 2 : 4-dinitro-phenylhydrazine. The product dissolves in strong alkali and gives a red colour which may be measured.

The estimation must be carried out immediately, since the concentration in shed blood rapidly falls, but this may be minimised if the blood is immediately mixed with mono-iodoacetic acid.

* Klein, D., *J. Biol. Chem.*, 1941, **137**, 311.

The normal concentration range for pyruvic acid in blood is from 0·7–1·2 mg. per 100 ml. The concentration of pyruvic acid in blood is increased when there is a deficiency of the co-enzyme containing the pyro-phosphoric ester of vitamin B_1, and is therefore considered significant of vitamin B deficiency.

REAGENTS.
1. *Iodoacetic acid, a 50% solution adjusted to pH 7·8 with caustic soda.*
2. *Sodium tungstate, 10% solution.*
3. *2 : 4-Dinitro-phenylhydrazine, a 0·5% solution in 2N hydrochloric acid.*
4. *Sulphuric acid, 2/3N.*
5. *Ethyl acetate.*
6. *Sodium carbonate, 1% solution.*
7. *Sodium hydroxide, 4N.*
8. *Potassium oxalate.*

METHOD. 0·05 ml. of 50% sodium iodo-acetate and 20 mg. of potassium oxalate are placed in a flask and 5 ml. of blood are run in directly. After mixing, 35 ml. of water, followed by 5 ml. of sodium tungstate, and 5 ml. of 2/3N sulphuric acid are added. The mixture is thoroughly shaken, allowed to stand for 15 minutes, and filtered. 10 ml. of the filtrate are transferred to a separating funnel, and 1 ml. of dinitro-phenylhydrazine is added. After standing for 15 minutes the mixture is extracted three times with 5 ml. of ethyl acetate. The combined extracts are washed with 2 ml. of water, and allowed to stand for 24 hours in the presence of water, in order to obviate the possible effects of other ketonic acids. The water is next removed and the hydrazone salt is extracted from the ethyl acetate with two successive 2 ml. portions of 1% sodium carbonate solution. Then the alkaline extract is shaken with 1 ml. of ethyl acetate to remove the excess of phenylhydrazine, transferred to a 10 ml. cylinder, and made up to 8·0 ml. with more sodium carbonate solution. 2·0 ml. of 4N sodium hydroxide solution are added, and the red colour is compared against standards.

(d) Estimation of Tartaric Acid*

PRINCIPLES. Tartaric acid modifies the yellow colour of *meta*-vanadic acid to red in a manner proportional to its concentration.

REAGENTS.
1. *Glacial acetic acid.*
2. *Sodium meta-vanadate, 5% solution.*
3. *Activated charcoal.*

METHOD. The sample, which should contain between 1 and 2 mg. of tartaric acid, is boiled with sufficient activated charcoal to remove residual colour.

* Underhill, F. P., Peterman, F. I., and Kraus, A. G., *J. Pharmacol.*, 1931, **43**, 351.

After filtration, the solution is neutralised and made up to about 50 ml. with water. 1 ml. of glacial acetic acid and 4 ml. of sodium *meta*-vanadate are added, and the colour reaction is allowed to occur. At first a greenish colour appears, but then the solution becomes red, reaching a maximum at about 10 minutes. After this, fading occurs at a slow rate. It is essential, therefore, to make colorimetric comparison after 10 minutes, preferably against a series of standards containing 0·2–2 mg. of tartaric acid, each of which has been treated in the manner described above.

(e) Estimation of Citric Acid*

PRINCIPLES. If citric acid is treated with sulphuric acid and bromine, penta-bromacetone forms. This may be extracted with ether, and on treatment with sodium sulphide produces an orange-red colour. The reaction is stabilised by addition of dioxane. 0·1 mg. may readily be estimated.

REAGENTS.

1. *Sulphuric acid, 50% solution.*
2. *Bromine water, saturated.*
3. *Potassium bromide, 1·0M.*
4. *Potassium permanganate, 1·5N.*
5. *Sodium sulphide, 4% solution, freshly filtered before use.*
6. *Purified dioxane.*†

METHOD. The sample, which should contain not more than 1 mg. of citric acid, is transferred to a beaker and made up to a volume of about 75 ml. 3 ml. of 50% sulphuric acid are added, and the mixture is boiled for 10 minutes to destroy substances which would subsequently interfere with the colour reaction. The mixture is then cooled, treated with 3 ml. of bromine water, and allowed to stand for 10 minutes. Any precipitate is removed by centrifuging, and the supernatant fluid is transferred to a separator. The concentration of sulphuric acid in the solution is increased to 1N.‡ 2 ml. of 1·0M potassium bromide and 10 ml. of 1·5N potassium permanganate are added, and the mixture is allowed to stand for 10 minutes to complete oxidation. Excess of permanganate is then destroyed by titrating with hydrogen peroxide at 6° C., an excess of which must be avoided. The solution is then extracted twice with 25 ml. of ether and the ether extract is twice washed with 5 ml. of water. The ether layer is then shaken successively with 3 ml., 2 ml., and 1 ml. of 4% sodium sulphide solution. The combined sulphide extracts are transferred to a 10 ml. tube containing 3·5 ml. of dioxane, and the whole is made up to 10·0 ml. with dioxane. The colour should then be read within 30 minutes.

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* Pucher, G. W., Sherman, C. C., and Vickery, H. B., *J. Biol. Chem.*, 1936, **113**, 235.
† Johnson, W. A., *Biochem. J.*, 1939, **33**, 1046.
‡ Dickens, F., *Biochem. J.*, 1941, **35**, 1011.

(f) *Estimation of Acetoacetic Acid**

PRINCIPLES. On account of enolisation ferric chloride gives a reddish-brown colour with acetoacetic acid. Salicylic acid can also be estimated with this reagent (compare p. 329).

REAGENT.
Ferric chloride, a neutral 15% solution.

METHOD. The solution, which should be neutral, is treated with one-fifth its volume of ferric chloride solution, and the red colour, which is produced immediately, is measured colorimetrically.

The method is not specific for acetoacetic acid, since phenols and other enolisable substances also give ferric chloride colours. A more accurate result is obtained if a further sample be treated with acid, boiled to decompose acetoacetic acid, then neutralised and again treated with ferric chloride. The concentration of acetoacetic acid is given by the difference between the two results.

(g) Estimations of Benzoic,† Hippuric, and Cinnamic Acids

PRINCIPLES. Benzoic acid is partially converted to salicylic acid by treatment with hydrogen peroxide. Addition of a ferric chloride reagent then produces a violet colour. This method may also be used for the estimation of both hippuric and cinnamic acids, if the former is hydrolysed and if the latter is previously oxidised (by neutral or alkaline permanganate) to benzoic acid.

REAGENTS.

1. *Ferric chloride reagent: for oxidation.* 2·5 g. anhydrous ferric chloride are dissolved in 13 ml. of N sulphuric acid and diluted to 100 ml. with water.

2. *Ferric chloride reagent: for colorimetric determination.* 0·1 g. anhydrous ferric chloride is dissolved in 20 ml. N hydrochloric acid and diluted to 100 ml. with water.

3. *Sulphuric acid, $N/10$.*

4. *Hydrogen peroxide, 0·2%.*

5. *Ammonium sulphate.*

6. *Ether-petrol.* Equal parts by volume of ether and light petroleum (b.p. 40°–60° C.).

7. *Phosphoric acid.*

METHOD. The sample containing benzoic acid is heated under a reflux with an equal volume of concentrated hydrochloric acid for about 30 minutes.

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* Snell, "Colorimetric Methods of Analysis" (Chapman and Hall, London, 1937), Vol. II, p. 119; Fearon, W., "Introduction to Biochemistry" (Heinemann, London, 1946), p. 463.

† Edwards, F. W., Nangi, H. R., and Hassau, M. K., *Analyst*, 1937, **62**, 172.

The cool solution is then extracted three times with 20 ml. of ethyl ether/ petroleum mixture. The solvent layer is treated with a few drops of strong ammonia. If benzoic acid was originally present in the sample, then a precipitate of ammonium benzoate will form. This is dissolved and extracted in 10 ml. of water. The extraction is twice repeated and the watery solutions are united. The ammonium benzoate solution is then acidified with phosphoric acid and steam distilled into about 10 ml. of N sodium hydroxide.

A portion of this solution, which should not contain more than 5 mg. of benzoic acid, is treated with 5 ml. of $N/10$ sulphuric acid and the volume is adjusted to 15 ml. with water. 1 ml. of the oxidation reagent (1) is added, followed by 1 ml. of dilute hydrogen peroxide. After shaking, the mixture is heated on a boiling water-bath for 15 minutes. The liquid, which is violet in colour, is cooled, about 5 g. of solid ammonium sulphate are added, and the salicylic acid is extracted with three successive quantities of 15 ml. of ether, each ether extract being washed with 5 ml. of water. The ether solution is then distilled on a water-bath at 50° C. and the dry residue is taken up in 50 ml. of 10% alcohol. 1 ml. of the ferric chloride reagent (2) for colour determination is now added, and the violet colour thereby produced is compared against standard solutions of salicylic acid which have been treated in an identical manner.

Since the conversion to salicylic acid is of the order of 10% only, a correction factor must be made. This is determined by trial for the particular concentration range of benzoic acid to be estimated.

(h) Estimation of Salicylic Acid (by Diazotisation)*

PRINCIPLES. Salicylic acid will couple with diazotised p-nitraniline to give a yellow azo dye, which may be measured colorimetrically. Other phenolic compounds and aromatic amines must, of course, be absent.

REAGENTS.

1. *Sodium acetate, 50%.*

2. *p-Nitraniline reagent.* 1·5 g. of p-nitraniline are dissolved in 40 ml. of concentrated hydrochloric acid and the solution diluted to 500 ml. with water. 25 ml. of this solution are mixed with 1 ml. of 7·5% sodium nitrite solution immediately prior to use.

3. *Sodium carbonate, 20% solution.*

METHOD. The salicylic acid is contained in 10 ml. of water. To this is added 1 ml. of 50% sodium acetate, followed by 1 ml. of diazotised p-nitraniline. After mixing and standing for 1 minute, 2 ml. of sodium carbonate solution are added, and colour comparison is made after 30 minutes.

* Theis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1924, **61**, 67.

(i) Estimation of Glycuronic Acid

Method 1: *Reaction with Orcinol.**

PRINCIPLES. If glycuronic acid is treated with orcinol a green colour is produced which is extractable with amyl alcohol. Glycuronic acid often occurs in urine in conjugated form, and a preliminary acid hydrolysis then becomes necessary before carrying out the estimation. The same reagent may be used for the estimation of pentoses.

REAGENT.
Bial's reagent. Add 1 g. of orcinol and 1·5 ml. of 10% ferric chloride to 500 ml. of concentrated hydrochloric acid.

METHOD. The solution of glycuronic acid is heated for 10 minutes in a boiling water-bath with four times its volume of Bial's reagent. After cooling, the green colour is extracted with amyl alcohol (one-fifth of the volume of the aqueous solution). The amyl alcohol layer is dried with sodium sulphate and then measured colorimetrically.

Method 2: *Reaction of Hexuronic Acids with Carbazole.†*

PRINCIPLES. Under appropriate conditions of acid concentration, hexuronic acids give with carbazole a pink colour which is specific.

REAGENT.
Carbazole. A 0·1% solution in alcohol.

METHOD. To 1 ml. of a solution containing 5–100 μg. of the hexuronic acid are added, with cooling, 6 ml. of concentrated sulphuric acid and the mixture is then heated on the water-bath for 20 minutes. After cooling, 0·2 ml. of the carbazole solution is added, drop by drop, with shaking. The pink colour which appears immediately and reaches a maximum intensity in two hours is then measured against standards in the usual way.

(k) Estimation of Tannic Acid‡

PRINCIPLES. In the presence of tartrate, ferrous sulphate reacts with tannic acid to form a blue-violet colour (a common form of ink).

REAGENT.
Ferrous tartrate solution. 0·1 g. of ferrous sulphate and 0·5 g. of sodium potassium tartrate are dissolved in 100 ml. of water.

METHOD. Tannic acid is extracted from the substance to be analysed by treating a washed sample with successive 150 ml. portions of water, boiling for an hour each time, and finally making up to a total volume of 500 ml.

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* Scheff, G., *Biochem. Zeit.*, 1927, **183**, 341.
† Dische, J., *J. Biol. Chem.*, 1946, **167**, 189.
‡ Mitchell, C. A., *Analyst*, 1923, **48**, 2.

An aliquot of this containing about 1 mg. of tannic acid is further diluted to 100 ml. with water and 2 ml. of ferrous tartrate reagent are added. The coloration thus produced is compared immediately against that given by a standard solution of gallic acid, or else is read in a photo-electric colorimeter for which an appropriate calibration curve has been made.

(l) Estimation of Barbiturates*

PRINCIPLES. The material (e.g. urine or blood) is dehydrated with sodium sulphate and extracted in a Soxhlet with ether-petroleum mixture. The purified extract is taken up with chloroform and treated with cobalt acetate in methyl alcohol and *iso*-propylamine to give a violet colour. Quantities of the order of 1 mg. can be estimated.

REAGENTS.
1. *Sodium sulphate, anhydrous.*
2. *Ether,* peroxide-free, mixed with an equal volume of *light petroleum.*
3. *Decolorising mixture* (magnesium oxide 1 part—charcoal 3 parts).
4. *Cobalt acetate,* 1% *in methyl alcohol.*
5. *Iso-propylamine,* 5% *in methyl alcohol.*

METHOD. The sample (e.g. 20 ml. of urine) is treated in a mortar with 2 g. of sodium dihydrogen phosphate. To this are added, small portions at a time, with grinding, 40 g. anhydrous sodium sulphate, and the mixture is then placed in a vacuum desiccator for about ½ hour to remove any unabsorbed moisture. The dried mix is then transferred to a Soxhlet thimble and extracted for about 3 hours with ether-petroleum mixture. The extract is then shaken with 0·25 g. of decolorising mixture, which removes phospholipins as well as colour. The ether solution is then filtered and transferred into a distilling flask, to which is added the ethereal washings from the decolorising mixture. The solvent is completely removed by distillation, and the residue is taken up in 10 ml. of chloroform. An aliquot of this solution, e.g. 2·0 ml., is treated with 0·1 ml. of cobalt acetate in methyl alcohol, followed by 0·6 ml. of *iso*-propylamine solution, and the violet colour, which is immediately produced, is compared against standard barbiturate solutions in chloroform which have been treated in a similar manner. It is best to prepare solutions containing from 0·5–2·5 mg. of barbituric acid for this purpose.

4. SUGARS

Methods (*a*)–(*c*) can be used generally for hexoses, but (*b*)–(*e*) need special calibration if applied to sugars other than glucose. Of these, (*a*) and (*c*) are fairly specific for glucose. Method (*d*), though very rapid, is not specific for sugars.

* Levvy, G. A., *Biochem. J.*, 1940, **34**, 73.

Methods (f) and (g) are specific for fructose, and can be used to differentiate between this sugar and glucose, though pentoses affect both reagents.   The carbazole reaction (e) is useful for the determination of conjugated sugars.

The reactions for pentoses (i, k) are general for sugars of this group and their derivatives (pentosans), but are not given by hexoses or rhamnose. The reaction for rhamnose (h) is not given by hexoses.

Method (b) can be used for maltose and lactose.   Sucrose gives none of these colour reactions without prior hydrolysis, but this substance, starch, and most other polysaccharides can be estimated by these methods if complete acid hydrolysis has been carried out.

(a) Estimation of Glucose (in Blood)* with Folin-Benedict Reagent

PRINCIPLES. Reducing sugars convert many cupric salts to the cuprous condition.   By using a special cupric bisulphite reagent the reduction can be made specific for glucose in blood.   The cuprous salt subsequently reduces phospho-tungstic acid to a blue complex which can be measured colorimetrically.

REAGENTS.

1. *Tungsto-molybdate solution.* Dissolve 10 g. of molybdic acid in 50 ml. of N caustic soda solution; boil the mixture for 5 minutes, filter, and pass 150 ml. of hot water through the filter-paper, adding this to the filtrate. Dissolve 80 g. of sodium tungstate in 600 ml. of water.   Mix the two solutions and complete to 1 l.

2. *Sulphuric acid,* $0·62N$.

3. *Copper reagent.* Dissolve 15 g. of anhydrous sodium carbonate, 3 g. of alanine, and 2 g. of Rochelle salt solution in 300 ml. of water.   Dissolve 3 g. of copper sulphate in 100 ml. of water.   The two solutions are mixed and diluted to 500 ml.   This solution should be renewed each month.

4. *Sodium bisulphite,* 1% solution.

5. *Phospho-molybdic reagent.* Dissolve 150 g. of molybdic acid and 75 g. of anhydrous sodium carbonate in 500 ml. of water, by boiling if necessary. Filter the solution, add the filtrate washings, and complete to 600 ml.   Then add 300 ml. of 85% phosphoric acid and dilute the solution to 1 l.

6. *Glucose solution.* Dissolve 1 g. of anhydrous glucose in 100 ml. of water and add a few drops of toluene as a preservative.   This solution is diluted 100 times before use.

METHOD. 5 ml. of tungsto-molybdate solution are transferred to a 250 ml. flask, 150 ml. of water and 5 ml. of $0·62N$ sulphuric acid are added, and the mixture is diluted to 250 ml.

5·0 ml. of this solution are transferred to a 15 ml. centrifuge tube and 0·1 ml. of blood is added. After mixing, the tube is set aside for 1 minute and then is centrifuged. 2·0 ml. of the clear supernatant fluid are transferred to a special tube (fig. IV.16) (e.g. a narrowed test-tube designed to prevent atmospheric re-oxidation) and 1·0 ml. of a solution prepared by mixing 2·0 ml. of bisulphite solution with 20 ml. of copper reagent is added. 1·0 ml. of distilled water should complete the volume to the constriction. At the same time a similar tube is prepared using 2·0 ml. of dilute glucose standard and copper reagent and water added to the same volume. The two tubes are heated in boiling water for exactly 5 minutes, cooled under running water for 1·0 minute, and 2·0 ml. of phospho-molybdate reagent are then added. The volume is diluted to 12·5 ml., mixed by inversion, and compared in the colorimeter after 10 minutes.

(b) Estimation of Glucose, Maltose, etc., with a Modified Folin-Benedict Reagent*

PRINCIPLES. The alkaline copper tartrate reagent of Folin and Benedict needs modification for other sugars. The new reagent of Somogyi has more general applicability; it is sufficiently alkaline to permit the determination of lactose, maltose, and other slowly reacting sugars, and because it contains no potassium iodide it is quite stable and shows no auto-reduction, even in sunlight. Again, on account of the high concentration of sulphate and the absence of iodide, reoxidation of cuprous oxide does not occur. The method for blood analysis is given below:
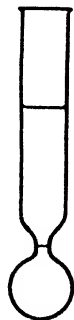
Fig. IV.16.
Sugar Tube.

REAGENTS.

1. *Barium hydroxide*, 0·3N.

2. *Zinc sulphate.* 5 g. of $ZnSO_4,7H_2O$ in 100 ml. of solution.

3. *Copper reagent.* 28 g. of anhydrous Na_2HPO_4, 100 ml. of N caustic soda, 40 g. of Rochelle salt, 8 g. of $CuSO_4,5H_2O$, and 180 g. of anhydrous Na_2SO_4 are dissolved in 1 l. of water. It is best to dissolve the salts separately and mix. The solution is allowed to age for 2 days and then is filtered.

4. *Arseno-molybdate reagent.*† Dissolve 25 g. of ammonium molybdate in 450 ml. of water. Add 21 ml. of concentrated sulphuric acid, and to this a solution of 3 g. of $Na_2HAsO_4,7H_2O$ dissolved in 25 ml. of water. Mix, and stand at 37° C. for 48 hours before use.

METHOD. 1 volume of blood is mixed with 5 volumes of water, and 2 volumes of barium hydroxide followed by 2 volumes of zinc sulphate solution are added. The mixture is shaken vigorously and filtered. To 2·0 ml. of the

* Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 69.
† Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

filtrate are added 2·0 ml. of copper reagent, the tube is covered and immersed in a boiling water-bath for 10 minutes, and, after cooling, 2·0 ml. of arseno-molybdate reagent are added. The solution, suitably diluted, is colori-metrically compared with standards.

(c) Estimation of Glucose (in Blood) by Reduction of Ferricyanide*

PRINCIPLES. Blood is freed from protein and other interfering substances by precipitation with colloidal zinc hydroxide. The filtrate is heated with alkaline ferricyanide and the ferrocyanide produced by oxidation of the glucose is measured colorimetrically as brown uranium ferrocyanide. Great precaution must be taken to avoid introducing reducing substances *via* dirty apparatus, hands, etc. (see pp. 6, 210, 267).

REAGENTS.

1. *Zinc sulphate,* 0·45% *solution.*

2. *Sodium hydroxide, N/10.*

3. *Potassium ferricyanide solution.* Wash the crystals with water to remove ferrocyanide and make a 0·6% solution. Store in a dark bottle.

4. *Cyanide-carbonate solution.* Dissolve 1·5 g. of iron-free sodium cyanide in 500 ml. of water and mix with an equal volume of 15% sodium carbonate solution.

5. *Uranium acetate solution.* Dissolve 10 g. of uranium acetate in water. Add 100 ml. of 2% gum ghatti solution (p. 291), 200 ml. of glacial acetic acid, and complete to 1 l. with water.

METHOD. Into a centrifuge tube containing 4·9 ml. of zinc sulphate solu-tion are pipetted 0·1 ml. of freshly shed blood and 1·0 ml. of $N/10$ sodium hydroxide solution. The tube is rotated to mix the contents thoroughly (avoid use of thumbs), placed in a boiling water-bath for 3 minutes, and the solution is then filtered through a pledget of well-washed cotton-wool placed in the apex of a small filter-funnel.

3·0 ml. of the filtrate are transferred to a clean, acid-washed test-tube, 2·0 ml. of the ferricyanide solution and 2·0 ml. of the cyanide reagent are added, and, after mixing the contents by rotating, the tube is placed in a *boiling* water-bath for exactly 10 minutes. The tube is then cooled under running water and 5·0 ml. of the uranium solution are added. The brown colour, which is produced immediately, is measured in a photo-electric colorimeter and the glucose concentration is noted from a calibration curve.

If a colorimeter is used, then the standard glucose solution must be treated in the same manner *at the same time as is the blood filtrate.* A standard glucose solution is prepared by dissolving 1 g. of anhydrous glucose

* Milton, R., *Analyst*, 1942, **67**, 183.

in 100 ml. of water. If a few drops of toluene are added the solution keeps for about 6 months. For colorimetric comparison 5 ml. of this solution are diluted to 500 ml. with water. The product then contains 0·1 mg. of glucose per millilitre, and with toluene will keep unchanged for about 2 months.

(d) Estimation of Hexoses by Reduction of Picric Acid*

PRINCIPLES. Reducing sugars are oxidised when heated with picric acid and sodium carbonate, and an orange colour, said to be due to picramic acid, is formed. Since polyphenols, purines, aldehydes, and similar oxidisable substances also give coloured solutions, the method is not specific, but can often be developed into a useful analytical procedure. It is capable of detecting 40 μg. of glucose.

REAGENTS.

1. *Picric acid, a saturated aqueous solution.*

2. *Sodium carbonate, 20% solution.*

METHOD. Solutions containing proteins (e.g. blood) are deproteinised by addition of saturated picric acid solution. Coloured solutions are decolorised by shaking vigorously with animal charcoal. Alternatively, clarification with basic lead acetate solution may be adopted, the excess of lead being removed by treatment with sodium phosphate.

To the clear solution is added 2 ml. of picric acid, followed by 1 ml. of 20% sodium carbonate solution.

The mixture is then heated on a boiling water-bath for 30 minutes and then, after cooling, compared against a standard solution of glucose which has been similarly treated.

(e) Estimation of Hexoses by means of Carbazole†

PRINCIPLES. A colour is produced when hexoses are heated with a solution of carbazole in sulphuric acid. Glucose and thymonucleic acid (compare p. 369) give red colours; other sugars give colours varying from red to brown. The method is sensitive to as little as 5 μg.

REAGENT.

Carbazole, in 80% sulphuric acid (by volume).

METHOD. The sugar solution is decolorised by treatment with charcoal or with lead acetate (see above). The solution is then heated with nine times its volume of carbazole solution and the colour compared against a similarly treated glucose standard.

* Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, **20**, 61.

† Dische, Z., *Mikrochemie*, 1930, **8**, 4.

(f) Estimation of Fructose with Resorcinol*

PRINCIPLES. A cherry-red colour is produced when fructose is heated with an alcoholic solution of resorcinol in hydrochloric acid. Pentoses should not be present.

REAGENTS.
1. *Resorcinol, 0·1% solution in 95% alcohol.*
2. *Hydrochloric acid, 30%.*

METHOD. Blood is deproteinised with zinc sulphate/sodium hydroxide (see p. 334). Urine is decolorised with acid-washed charcoal in acetic acid solution.

To 2·0 ml. of the clear solution are added 2·0 ml. of resorcinol solution, followed by 6·0 ml. of 30% hydrochloric acid solution. After mixing, the tube is placed in a water-bath at 80° C. for 8 minutes. The cooled solution is then compared against a similarly treated standard solution of fructose (0·1 mg. in 2 ml. of water).

(g) Estimation of Fructose with Orcinol†

PRINCIPLES. A very sensitive reaction occurs between orcinol and small amounts of fructose, giving a yellow precipitate which will dissolve in alkali to give an orange colour. A 0·005% solution of fructose may be estimated in this way. Glucose does not react, but pentoses also give colours.

REAGENTS.
1. *Orcinol, 0·2% in water.*
2. *Syrupy phosphoric acid, 85%.*
3. *Caustic soda, 5N.*

METHOD. A protein-free, clear solution containing fructose is treated with 0·4 volume of aqueous orcinol solution followed by 1 ml. of syrupy phosphoric acid. The mixture is first heated over a free flame to boiling, and then is transferred to a boiling water-bath for 10 minutes. The solution is then transferred quantitatively to a 10 ml. standard flask and completed to the mark with 5N caustic soda. While still warm it is compared visually against a similarly treated standard solution of fructose.

(h) Estimation of Rhamnose with Phloroglucinol‡

PRINCIPLES. On acid hydrolysis rhamnose yields methyl furfural. This reacts with phloroglucinol in benzene solution to give a deep yellow product suitable for colorimetric estimation. Pentoses must not be present, since they would give a black colour.

* Roe, J. H., *J. Biol. Chem.*, 1934, **107**, 15.
† Loewe, L., *Proc. Soc. Exp. Biol. Med.*, 1916, **13**, 71.
‡ McCance, R. A., *Biochem. J.*, 1929, **23**, 1172.

REAGENTS.

1. *Hydrochloric acid, 1 : 1 solution.*

2. *Phloroglucinol, 0·25% solution in 95% alcohol.*

METHOD. The sample is diluted to contain about 1 mg. of the sugar per millilitre. 3 ml. of this solution and 6 ml. of 1 : 1 hydrochloric acid are refluxed on a boiling water-bath for 2 hours. The methyl furfural is then extracted by shaking the cooled solution with 4 ml. of benzene. The extract is allowed to stand for 20 minutes and then is centrifuged.

2·0 ml. of the benzene layer are transferred to a dry test-tube, 4·0 ml. of the phloroglucinol solution are added and then, with shaking, 0·65 ml. of concentrated hydrochloric acid. The yellow colour, which forms in under 3 minutes, is completely developed after 30 minutes and then can be matched against a standard.

(i) Estimation of Pentoses as Furfural by Reaction with Benzidine*

PRINCIPLES. On hydrolysis, pentoses such as arabinose and xylose are split to yield furfural. This may be extracted with benzene and made to react with benzidine to give a reddish-violet colour, a distillation of the furfural being unnecessary.

REAGENTS.

1. *Hydrochloric acid, 1 : 1.*

2. *Benzidine, 0·5 g. dissolved in 50 ml. of absolute alcohol to which has been added 50 ml. of glacial acetic acid.*

METHOD. 3 ml. of an aqueous solution of a pentose (containing *ca.* 1 mg. per millilitre) are transferred to a test-tube and 6 ml. of 1 : 1 hydrochloric acid are added. The tube is fitted with an air condenser and is heated on a boiling water-bath for 2 hours. At the end of this time the tube is cooled, any condensate is rinsed into the tube, and the whole is extracted by shaking for about 3 minutes with 4 ml. of benzene. The benzene layer is then transferred to a centrifuge tube and the liquid is centrifuged until clear. Exactly 2·0 ml. of the clear layer are then transferred to a dry test-tube, 4·0 ml. of the benzidine reagent are added, and the contents are mixed. The colour takes about 15 minutes to develop fully.

It is essential that comparison should be made against a standard solution of the pentose which has been treated similarly, since the transformation to furfural is never complete and depends to some extent upon the conditions of the experiment.

(k) Estimation of Pentoses as Furfural by Reaction with Xylidine†

PRINCIPLES. Pentosans and pentoses are hydrolysed to furfural which is separated by steam distillation. Reaction of the distillate with xylidine

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* McCance, R. A., *Biochem. J.*, 1926, **20**, 1111.
† Suminokara, K., *J. Biochem. Japan*, 1931, **14**, 343.

leads to the formation of a red colour suitable for comparison.   Colourless, *freshly distilled*, aniline may be used in place of xylidine.

REAGENTS.

1. *Sulphuric acid, 12·5N, saturated with potassium sulphate.*

2. *Zinc sulphate.*

3. *Xylidine reagent.* 10·0 ml. of pure (i.e. colourless) *m*-xylidine in 50 ml. of glacial acetic acid.

METHOD. The solution of pentosans or pentoses containing not more than 20 mg. of substance is transferred to a distilling flask, 30 ml. of sulphuric acid/potassium sulphate mixture and 2 g. of zinc sulphate are added, and connection is made to a condenser system.   The air in the apparatus is removed by passage of a current of carbon dioxide.   The flask is then placed in an oil-bath which is gradually heated to 150° C.   The current of carbon dioxide is maintained, and the rate of steam distillation is controlled so that 90 ml. of liquid condense in about 25 minutes.   This is usually sufficient for the removal of all the furfural, but it is advisable to continue distillation until no colour is given on testing a further measured sample with xylidine reagent.

10 ml. of the distillate are treated with 15 ml. of the xylidine reagent, and the colour is compared after allowing 30 minutes for development.

For comparative purposes it is best to use standard solutions of freshly distilled furfural, choosing concentrations of the order of 0·01 mg.   Each of these must be developed with the xylidine mixture at the same time as the unknown sample.   If this method is followed, then a conversion factor must be used according to the particular pentose present:

Conversion factor of furfural value.

Arabinose: 1·80
Xylose: 1·61
Pentoses: 1·70 (usual mixture)
Pentosans: 1·50 (usual mixture).

5. PHENOLS

The most direct method of estimating phenols (*a*) depends upon the fact that they can be coupled with diazonium salts to give azo dyes.   Diazotised sulphanilic acid and diazotised *p*-nitraniline are the reagents most commonly used.   Since amines, proteins, and their decomposition products may also yield azo dyes, Folin's phenol reagent (*b*) is more convenient for biochemical use and gives the same colour with all phenols.   However, it will react with many other substances, e.g. purines, thiols, and many reducing agents such as glucose.   The indophenol dye reaction (*c*) is rather more specific for free

phenols in the absence of oxidising and reducing agents, and is particularly suited for water analysis. Millon's reagent, which was also used for this purpose,* is less reliable. This is indicated in another connection, on p. 350.

(a) Estimation of Phenols Using Diazotised Sulphanilic Acid†

PRINCIPLES. Diazotised sulphanilic acid couples with phenols, imidazoles, and with some aromatic amines. The coupling occurs preferentially in the *para* position, but when this is occupied, the *ortho* position is involved. With phenol itself a greenish-yellow colour is obtained; most other phenols give reddish colours.

One part of a phenol in 10 million of water can be estimated by this method, which was first developed for the examination of river-water. Diazotised *p*-nitraniline can also be used as the coupling agent (compare p. 329).

REAGENTS.

1. *Sulphanilic acid, an* 8% *solution of recrystallised acid in* 0·25% *hydrochloric acid.*

2. *Sodium nitrite,* 8% *solution.*

3. *Sodium hydroxide,* 10% *solution.*

METHOD. To the phenol in an ice-cold 50 ml. sample (e.g. a steam distillate) are added 4 ml. of sulphanilic acid reagent, followed by 2 ml. of sodium nitrite solution. After thorough mixing, 5 ml. of 10% sodium hydroxide are added. The colour is fully developed in 3 minutes, after which time the colorimetric comparison should be made without further delay. The standard phenol used for comparison purposes should be identical with the one which is being estimated, since different phenols yield colours of greatly differing tones.

(b) Estimation of Phenols Using Phosphotungsto-Molybdic Acid‡

PRINCIPLES. The complex mixture produced when phosphoric, molybdic, and tungstic acids are boiled is very unstable, and is readily reduced in alkaline solution to a blue colloid. A large range of substances will effect this reduction, but with certain previous precautions, the method may be used for the estimation of phenols. 1 part per million of phenol can be detected.

REAGENTS.

1. *Folin's phenol reagent.* Dissolve 100 g. of sodium tungstate and 25 g. of sodium molybdate in 700 g. of water, and then add 50 ml. of 85% syrupy phosphoric acid and 100 ml. of concentrated hydrochloric acid. Reflux the mixture for 10 hours in an all-glass apparatus. At the end of this time add 150 g. of lithium sulphate followed by 50 ml. of water and 0·5 ml. of

* Bach, H., *Z. Anal. Chem.*, 1911, **50**, 736.

† Fox, J. J., and Gauge, A. H., *J. Soc. Chem. Ind.*, 1920, **39**, 260T.

‡ Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.

bromine.   Then reboil the mixture for about 15 minutes to remove excess of bromine.   After cooling and dilution to 1 l., filter the reagent before using.

2. *Sodium carbonate*, 20% *solution.*

3. *Zinc chloride*, 2·5% *solution.*

4. *Sodium tungstate*, 10% *solution.*

METHOD.

(*a*) *Purification of Solution.* The solution containing phenol should be freed from interfering substances as completely as possible.   If protein is present (as in blood, etc.), 1 volume is diluted with 7 volumes of water, 1 volume of 10% sodium tungstate is added, and then 1 volume of 0·66N sulphuric acid.   This mixture is filtered, after shaking and standing for 1 hour, and the filtrate is then treated with one-tenth of its volume of 2·5% zinc chloride and one-tenth of its volume of 10% sodium carbonate solution, which precipitate purine bodies.   Phenol may now be estimated on the filtrate.

(*b*) *Estimation of Free Phenols.* To about 10 ml. of a solution which has been treated as above are added 5 ml. of Folin's phenol reagent, followed by 15 ml. of 20% sodium carbonate solution.   The mixture is then diluted to 50 ml. and held in a water-bath at 35° C. for 20 minutes to develop the colour completely.

(*c*) *Estimation of Total Phenols (Free and Conjugated) in Biological Samples.* The filtrate [as in (*a*) above] is acidified with a few drops of concentrated hydrochloric acid, brought to boiling, and refluxed for about 20 minutes. The cooled solution is neutralised with alkali, and then 5 ml. of the Folin reagent and 15 ml. of 20% sodium carbonate are added.   After diluting to 50 ml. with water, the mixture is maintained at 35° C. for 20 minutes, and then the colour comparison is made against standard solutions containing phenol in concentrations of the order of 0·025 mg. per millilitre.

(c) Estimation of Phenol by the Indophenol Reaction*

PRINCIPLES.   Most phenolic compounds which are unsubstituted in the *para* position combine with 2 : 6 dibromoquinonimide to give, in alkaline solution, a blue indophenol dye sensitive to 1 in 100 million.   Since the product is an indicator, it is essential that the colour comparison be made in a highly buffered solution at a given pH, e.g. pH 9·6.   Moreover, since indophenols are also oxidation-reduction indicators, the solution must be free from oxidising and reducing substances.   Many aromatic amines give indamine dyes by this treatment, but their colours are much less intense.

REAGENTS.

1. 2 : 6 *Dibromoquinonimide.* This specific reagent is prepared by the oxidation of 2 : 6 dibromo-4-aminophenol with sodium hypochlorite.   The

* Gibbs, H., *J. Biol. Chem.*, 1927, **72**, 649.

sodium hypochlorite is freshly prepared by dissolving 630 g. of sodium hydroxide in a little water and adding lumps of ice until the weight is about 10 kg. Chlorine gas is now bubbled into the mixture until the weight increases by another 730 g. 670 ml. of 2 : 6 dibromo-4-aminophenol are dissolved in cold dilute hydrochloric acid, and this solution is added to the cold hypochlorite solution, with stirring. Excess of aminophenol is to be avoided, since if no free chlorine is present in the solution a blue coloration will be produced at once. The chlor-quinonimide which separates off as a yellow solid is filtered, washed with a little water, and air-dried. The watery solution is unstable and an 0·3% solution in alcohol is prepared from the dry powder and extemporaneously diluted ten times with water.

2. *Buffer, pH* 9·6. 50 ml. of $M/5$ anhydrous boric acid in $M/5$ potassium chloride are mixed with 36·85 ml. of $N/2$ sodium hydroxide.

METHOD. The phenol solution is concentrated and separated by distillation as on pp. 339–340. The sample should contain about 1 mg. of phenol per 100 ml. 5·0 ml. of this solution are treated with 5·0 ml. of buffer solution and diluted to 100 ml. with water. 2 ml. of 0·03% quinonimide reagent are then added, and, after standing for 30 minutes, the tint is compared with that of a similarly treated standard. If a low phenol concentration is encountered (0·005 mg. per 100 ml.) a longer development period is required, and in this case auto-oxidation must be guarded against.

The presence of sulphuretted hydrogen in the distillate (which may interfere due to its reducing effect) can be counteracted by addition of 1 ml. of $N/1,000$ potassium ferricyanide to both standard and unknown solution.

6. HALOGEN COMPOUNDS

Estimation of Chloroform* and Analogues

PRINCIPLES. Several organic halogen compounds react with pyridine and alkali to give coloured complexes. Besides chloroform, iodoform, carbon tetrachloride, and trichlorethylene can also be estimated.

The procedure is convenient for vapour analysis and for estimation of chloroform, etc., in biological material. 1 part per million of chloroform can be detected.

REAGENTS.

1. *Tri-n-butyl citrate.*
2. *Toluene, pure redistilled.*
3. *Pyridine, pure.*
4. *Sodium hydroxide*, 20% *solution.*

METHOD. The sample (e.g. 25 ml. of blood) is made up to 100 ml. with distilled water and transferred to an all-glass distillation apparatus. Foaming

* Habgood, S., and Powell, J., *Brit. Jour. Ind. Med.*, 1945, **2**, 39.

is controlled by the addition of a few drops of tri-*n*-butyl citrate. The receiver, a 50 ml. measuring cylinder with ground-glass neck, is cooled in ice water and contains about 1·5 ml. of toluene. Distillation is allowed to proceed until about 40 ml. have been collected, and then the condenser tube is washed down with a little water. The measuring cylinder is stoppered and shaken. After stirring, the toluene layer is pipetted off.

For the colour reaction 1 ml. of toluene extract is added to 10 ml. of pyridine and then 5 ml. of 20% caustic soda. After heating at 100° C. for exactly 5 minutes the mixture is cooled and the pyridine layer is separated off. (Prolonged contact with alkali causes colour fading.) At this stage the pyridine solution is often turbid, but may be cleared by the addition of water to bring the volume up to 15 ml. The colour can be measured in a Pulfrich photometer using filter S 53, but it can also be estimated in a Spekker absorptiometer fitted with a green screen or in a colorimeter against standard solutions prepared from the appropriate chloro-hydrocarbon. With carbon tetrachloride and chloroform the pyridine layer exhibits a purplish-red colour, whilst trichlorethylene gives an orange-red.

7. HYDROCARBONS

Estimation of Benzene*

Example: Vapour Estimation.

PRINCIPLES. If benzene is dinitrated the product when extracted with butanone and treated with alkali produces a bluish-red colour. A similar colour is given by toluene, and a green colour by xylene. If, however, the alkaline butanone extract be acidified, then the colours due to xylene and toluene are destroyed and the method becomes specific for benzene. An excess of sulphuric and fuming nitric acids is used for nitration, since this will quantitatively dinitrate benzene in 30 minutes. In the absence of sulphuric acid the nitration is varied and incomplete and there is a tendency to form a mono-nitro compound.

The technique described will readily measure benzene in the presence of twenty times its concentration of xylene or toluene. If the concentration of these substances is much higher, then the yellowish-brown colour due to their nitration products is so large as to make the difference readings questionable.

REAGENTS.

1. *Nitration mixture, equal volumes of concentrated sulphuric and fuming (d. 1·5) nitric acids.*

2. *Caustic soda, 40% solution.*

3. *Butanone (methyl ethyl ketone), pure redistilled.*

4. *Glacial acetic acid.*

* Milton, R., *Brit. Jour. Ind. Med.*, 1945, **2**, 36.

METHOD.

(a) *Vapour Sampling Procedure.* An absorption bubbler with beads to break the air stream is charged with 10 ml. of nitration mixture (see fig. IV.14, p. 300). The atmosphere is aspirated through the solution at a rate of 1 l. per minute, since at this speed absorption of benzene from the air is quantitative. At 5 l. per minute or more, there is a tendency to lose about 5% of the benzene, but this may be overcome if two bead bubblers are coupled in series. In the absorption train the bubbler is followed first by an absorption bottle containing 40% caustic soda to absorb nitric fumes aspirated from the acid mixture, and then by a flowmeter to indicate the rate of sampling. The apparatus can be operated by an air injector of the Venturi type attached to a compressed air bottle. When very low concentrations of benzene are present, a smaller absorber containing 2 ml. of mixture must be used.

(b) *Analysis.* After taking the sample, an interval of 30 minutes should elapse to complete the nitration. The sample may, however, be left at this stage for an indefinite period without any deterioration.

2·0 ml. of the acid mixture from the bubbler are transferred to a 50 ml. Erlenmeyer flask, diluted to 10 ml. with water, and neutralised with 40% caustic soda. Care should be taken to see that the solution is *just* alkaline to litmus, since the final colour is impaired if the solution is left acid, and if it is too alkaline premature colour development occurs.

10 ml. of pure butanone are then added and the mixture is shaken over a period of 10 minutes. The butanone layer is separated, removed to a test-tube, and diluted with 30 ml. of absolute alcohol. By this procedure extraction of the benzene compound is complete, although in the presence of xylene and toluene the yellow colour is distributed between the two layers. If much toluene and xylene has been present then the butanone is coloured yellowish-brown and the colour persists after making alkaline and subsequently acidifying. Consequently the optical density of the butanone-alcohol extract must be measured and an allowance made accordingly. If coloured, the butanone solution should be transferred to a Spekker absorptiometer fitted with a green screen and a reading taken (reading 1). If the solution is colourless at this stage, then this reading may be dispensed with.

The alcohol-butanone solution is next mixed with 0·1 ml. of 40% caustic soda solution. In the presence of benzene a red colour develops fully within 5 minutes. A purple or green colour, with varying shades of intensity according to the mixture, shows up if toluene or xylene have been present. The amount of soda to be added is not critical, but the bulk of water in the solution must be kept to a minimum in order to ensure homogeneity. 5 minutes are allowed for the alkaline colour to develop completely. 0·1 ml. of glacial acetic acid is then added and the whole is mixed. The alkaline colours due to xylene and toluene are completely destroyed, leaving a reddish

colour due to dinitrobenzene and the residual yellow colour of the butanone extract. The destruction of the xylene and toluene colour is immediate, and so the final Spekker reading may be made forthwith. Again the measurement is made through the green screen. The difference between this reading and that of the initial butanone extract represents colour due to benzene. The colour of the acidified solution is stable, and so the reading may be deferred for some hours if necessary. The result is referred to a calibration curve constructed as follows:

50 mg. of benzene are weighed in a closed vessel and dissolved in 100 ml. of absolute alcohol. 1 ml. of this solution will contain 500 μg. of benzene. From this solution five dilutions in absolute alcohol are made, giving samples containing 100, 200, 300, 400, and 500 μg. of benzene per millilitre. By means of a specially calibrated pipette (see p. 147) 0·1 ml. of each of these solutions is added to 2 ml. of the nitration mixture, allowed to stand for 30 minutes, and then treated as described above to develop the colour. The reading differences are plotted against concentration. This gives a calibration curve covering the sensitive range of the method.

8. AMIDES AND AMINO-ACIDS

Urea can be estimated quite simply by hydrolysis with urease (see p. 162) distillation, and subsequent estimation of ammonia with Nessler's solution (p. 297).

The exceedingly sensitive method of Milton and Obermer (*a*) is specially suitable for the estimation of urea in the presence of ammonium salts and amino-acids, both of which are found in urine. The xanthydrol method (*b*) is also selective and is more suitable for blood analysis.

Diazotisation followed by coupling with a phenol (e.g. H-acid) can be used for the estimation of all aromatic primary amines, the procedure being analogous to that of section 5 (*a*) (p. 339). The special application to sulphonamides (*s*) may be noted. For the estimation of *aniline* the hypochlorite colour reaction (*d*) (p. 347) is much more sensitive. Dimethylaniline, aniline, and analogous compounds can, of course, be coupled with diazonium salts.

Of the two general methods (*e, f*) for the estimation of *amino acids*, the ninhydrin reaction suffers from the defect that urea also reacts. Method (*g*) is specific for glycine.

Tyrosine and *tryptophane* can both be estimated by Folin's phenol reagent* (see p. 339), whilst tyrosine and *tyramine* can be estimated by coupling with diazotised sulphanilic acid (p. 339),† but Millon's reagent (*h*) is also useful

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* Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.

† Hanke, M. T., Milton, T., and Koessler, Karl K., *J. Biol. Chem.*, 1922, **50**, 235.

in this case. *Tryptophane* alone can be estimated with Ehrlich's reagent and nitrous acid (*i*).

Histidine also may be estimated by coupling with diazotised sulphanilic acid, but the bromination reaction (*k*) is more selective. *Cysteine* can be estimated with *o*-benzoquinone (*l*) or by the methylene-blue reaction given on p. 311.* The nitroprusside reaction is not suitable for quantitative colorimetry.

Creatine and *creatinine* are best estimated by their reduction of alkaline sodium picrate (*m*). Creatinine can also be estimated with 3 : 5-dinitrobenzoic acid,† but the colour is transient.

The estimation of *pyrrole* (*p*) illustrates the isatin reaction for heterocyclic bases, and that for *hydroxylamine* (*q*) the reactions of hydroxamic acids.

(a) Estimation of Urea in Urine with Stannous Chloride and Furfural‡

PRINCIPLES. The reaction between stannous chloride and furfuraldehyde in the presence of a mineral acid to produce a coloured condensation product is catalysed by urea. The rate of catalysis is proportional to the urea concentration. The condensation, which is continuous, is stopped after a proper time interval by the addition of a buffer which raises the *p*H.

REAGENTS.

1. *Stannous chloride, a* 10% *solution in concentrated hydrochloric acid.* (Store in a refrigerator.)

2. *Furfural solution.* 0·3 ml. of freshly distilled furfuraldehyde and 7 ml. of glacial acetic acid are made up to 21 ml. with 5% gum ghatti solution.

3. *Sodium acetate,* 30% *aqueous solution.*

4. *Lead acetate, a* 5% *solution in* 10% *acetic acid.*

5. *Sodium sulphate,* 10% *solution.*

6. *Gum ghatti,* 5% *solution* (see p. 291).

METHOD. 0·2 ml. of urine is pipetted into a test-tube containing 1 ml. of 10% stannous chloride in concentrated hydrochloric acid. To this is then added 0·3 ml. of the furfuraldehyde solution. After mixing, the tube is allowed to stand at room temperature for about 30 minutes. 45 minutes should be allowed if the room temperature is below 10° C. and only 20 minutes if it is above 20° C. The condensation reaction, as evidenced by the intense purple colour, is then arrested by the addition of 4 ml. of a mixture of 3 volumes of 30% sodium acetate and 1 volume of 5% gum ghatti. The purple colour changes from brownish-green to golden-brown, and the tint is stabilised after 30 minutes. It is then compared in a colorimeter against standard solutions of urea which have been similarly treated. In view of

* Vassel, B., *J. Biol. Chem.*, 1941, **140**, 323.
† Benedict, S. R., and Behre, J. A., *J. Biol. Chem.*, 1936, **114**, 515.
‡ Milton, R., and Obermer, E., *Analyst*, 1938, **63**, 423.

the fact that the colour intensity is not directly proportional to the urea concentration, it is essential to prepare a series of standards containing from 0·5–4% of urea, and compare against the nearest colour at the same time that the urine solution is being tested.

In the presence of over 0·5% of protein the urine will cause turbidity due to precipitation, and so make colorimetric comparison impossible. In this case the protein is removed by adding to 2 ml. of urine 0·5 ml. of lead acetate and 0·5 ml. of 10% acetic acid. The mixture is centrifuged and 2·0 ml. of the clear supernatant fluid are transferred to another centrifuge tube and treated with 0·5 ml. of 10% sodium sulphate to remove excess of lead. 0·2 ml. of this solution is then estimated for urea, a dilution factor of 1·875 being applied to the result.

Highly pigmented urines are clarified by shaking with absorbent charcoal in acid solution.

If the method is used in conjunction with the photo-electric absorptiometer it is essential that standards be prepared at the same time as the unknown, since the depth of colour is affected by variations in time and temperature.

(b) Estimation of Urea (in Blood) with Xanthydrol*

PRINCIPLES. Urea is precipitated from deproteinised blood with xanthydrol to produce dixanthylurea. The precipitate is dissolved in concentrated sulphuric acid giving an intense yellow colour.

REAGENTS.

1. *Trichloracetic acid*, 20%.
2. *Xanthydrol, a 5% solution in methyl alcohol.*
3. *Sulphuric acid, 50% by volume.*

METHOD. 0·5 ml. of blood is pipetted into a centrifuge tube containing 1·5 ml. of distilled water, 0·5 ml. of trichloracetic acid is added, and, after mixing, the tube is centrifuged. The supernatant fluid is poured through a filter-paper into a dry tube, 0·5 ml. of the clear filtrate transferred to another centrifuge tube, and 1·0 ml. of glacial acetic acid is added. To this mixture is then added 0·2 ml. of xanthydrol solution; the product is allowed to stand for 5 minutes, then is thoroughly agitated by tapping the bottom of the tube, and again is left to stand for a further hour. The tube is then centrifuged, the supernatant fluid is removed, and the precipitate is washed three times with 2 ml. portions of methyl alcohol saturated with dixanthylurea. The tube is finally drained by inversion over a filter-paper and the lip is wiped with a spill of filter-paper. The precipitate in the bottom of the tube is then loosened by tapping and dissolved in 5 ml. of 50% sulphuric acid. The yellow solution is compared in the colorimeter against a standard solution of dixanthylurea in 50% sulphuric acid.

* Beattie, F., *Biochem. J.*, 1928, **22**, 711.

(c) Estimation of Uric Acid (in Blood)*

PRINCIPLES. Uric acid lowers the reduction potential of a specially prepared phosphotungstic reagent sufficiently to produce a blue colour.

REAGENTS.

1. *Uric acid reagent.* 100 g. of sodium tungstate (free from molybdate) are transferred to a 100 ml. flask and to this is added a mixture of 33 ml. of 85% phosphoric acid with 100 ml. of water. The mixture is then gently boiled over a micro-burner for 1 hour, using an all-glass refluxing apparatus. The solution, which is deep green, is decolorised by the addition of a little bromine water and the excess of bromine is boiled off. About 3 g. of sodium tungstate are then added, boiling is continued for a further 15 minutes, and decolorisation with bromine is carried out as before. The volume is subsequently made up to 250 ml. with water.

2. *Cyanide-urea reagent.* 75 g. of sodium cyanide are dissolved in 700 ml. of water, 300 g. of urea are added, and the mixture is stirred until solution results. The reagent is freed from carbonate by the addition of 5 g. of calcium oxide, and after standing for about 24 hours is filtered. To the filtrate are added 2 g. of lithium oxalate; and after shaking for 15 minutes, it is filtered.

3. *Uric acid standard.* 0·6 g. of lithium carbonate is dissolved in 150 ml. of water warmed to 60° C.; the warm solution is poured on to 1 g. of uric acid in a 1 l. volumetric flask, and is shaken until the substance is dissolved. 20 ml. of 40% formaldehyde solution and 500 ml. of water are added to the solution. A few drops of methyl orange indicator are added, and N sulphuric acid (about 25 ml.) is run in until the colour changes to pink, and the whole is then diluted to 1 l. with water. This stock solution is quite permanent and 1 ml. is diluted to 250 ml. with water before use. 5 ml. of this solution are equivalent to 0·02 mg. of uric acid.

METHOD. 5 ml. of unlaked blood filtrate (see p. 333) are transferred to a test-tube with a graduation at 25 ml. 5 ml. of uric acid standard are placed in another tube and each tube is treated, from a burette, with 10 ml. of cyanide-urea solution. The additions to the tubes are mixed by a whirling motion. 4 ml. of uric acid reagent are added to each of the tubes, and after 30 minutes the samples are diluted to 25 ml. and compared.

(d) Estimation of Aniline by Calcium Hypochlorite† (Application to the Estimation of Aniline in Atmospheres)

PRINCIPLES. In dilute solution aniline gives a stable yellow colour with an alkaline solution of calcium hypochlorite. 0·5 part per million can be detected.

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* Folin, O., *J. Biol. Chem.*, 1933, **101**, 111.
† Pamfilov, A. V., *Ind. Eng. Chem.*, 1926, **18**, 763; Elvove, E., *Ind. Eng. Chem.*, 1917, **9**, 953.

REAGENTS.

1. *Sulphuric acid, 0·1N.*
2. *Sodium hydroxide, 0·1N.*
3. *Calcium hypochlorite solution, freshly prepared.*
4. *Sodium hydroxide, N solution.*

METHOD. Aniline vapour is absorbed by aspirating the air sample through 100 ml. of 0·1N sulphuric acid. 100 ml. of 0·1N sodium hydroxide are added, and a suitable dilution is made so that 20 ml. contain about 1 mg. of aniline. 20 ml. of the sample are then treated with 1 ml. of calcium hypochlorite solution and allowed to stand for 2 minutes. 1 ml. of N sodium hydroxide is added, and the mixture is allowed to stand for 10 minutes. For comparison a standard solution containing 1 mg. of aniline in 20 ml. of water is similarly treated.

(e) Estimation of Amino-acids with Sodium-β-Naphthoquinone Sulphonate*

Example: Estimation of Amino-acids in Blood.

PRINCIPLES. Amino-acids (and ammonia) give with sodium-β-naphthoquinone-4-sulphonate a red colour which under suitably controlled conditions is proportional to the concentration of the amino compound. Interfering proteins should be precipitated with tungstic acid.

REAGENTS.

1. *Sodium tungstate, 10%.*
2. *Sulphuric acid, 0·66N.*
3. *Sodium carbonate, 0·1% solution.*
4. *Hydrochloric acid, N/10.*
5. *Sodium-β-naphthoquinone sulphonate, 0·5% aqueous solution, freshly prepared.*
6. *Acetate buffer, equal volumes of 50% acetic acid and 5% sodium acetate.*
7. *Sodium thiosulphate, 4%.*

METHOD. 7·0 ml. of distilled water are mixed with 1·0 ml. of blood. After laking, 1·0 ml. of sodium tungstate and then 1·0 ml. of 0·66N sulphuric acid are added. The mixture is shaken very thoroughly, allowed to stand for 1 hour, and then filtered.

5·0 ml. of the filtrate are transferred to a test-tube, followed by a few drops of phenolphthalein. The mixture is acidified and then sodium carbonate is added drop by drop until a just perceptible pink colour persists.

At the same time a standard glycine solution (0·035 mg. of amino N in 5 ml.) is treated in the same way and made alkaline to exactly the same pink tinge. To each tube are then added 2·0 ml. of freshly prepared Folin's

reagent (solution 5) and then 1·0 ml. of 0·1% sodium carbonate solution. The tubes are stoppered and placed in a dark cupboard for 22 hours. At the end of this period 2·0 ml. of acetate buffer solution are added to each tube, followed by 1·0 ml. of thiosulphate solution, which destroys the colour due to the excess of the reagent. After suitable dilution the colours are compared.

(f) Estimation of α-Amino-acids Using Ninhydrin*

PRINCIPLES. α-Amino-acids on treatment with triketohydrindine hydrate (ninhydrin) give a permanganate-like coloration. The method is suitable for the estimation of 0·005–0·05 mg. per millilitre. When larger quantities of solution are available the gasometric method should be used (p. 556).

REAGENTS.
1. *M*/15 *Potassium dihydrogen phosphate*, 9·70 g. per litre.
2. *M*/15 *di-sodium hydrogen phosphate*, 11·86 g. per litre.
3. *Ninhydrin*, 1% solution, freshly prepared.
4. *Neutral red*, 0·01% solution in 50% alcohol.

METHOD. 2·0 ml. of the amino-acid solution to be analysed, 2·0 ml. of a standard amino-acid solution, and 2·0 ml. of a mixture of 2 parts of *M*/15 potassium phosphate and 3 parts of *M*/15 sodium phosphate (*p*H 6·97) are placed in test-tubes. 1 drop of 0·01% neutral red solution is added to each.

N/250 acid or alkali is then added to both the standard and unknown samples until the colour in the two tubes is exactly identical with that in the tube containing the buffer solution. (If there is a large difference in *p*H between the buffer and the unknown, then stronger acid or alkali should be used at first, the object being to keep the dilution as low as possible.) To each tube are then added 2·0 ml. of the buffer solution, followed by 1·0 ml. of ninhydrin solution. The tubes are then placed in a boiling water-bath for 1 hour, allowed to stand at room temperature for 1 hour, and then compared in a colorimeter. A satisfactory standard for the above purpose corresponds to 3 mg. of amino-acid per litre.

(g) Estimation of Glycine (in Blood)†

PRINCIPLES. Glycine is converted by ninhydrin to formaldehyde, which is then made to react with chromotropic acid to produce a colour. The method will measure 0·2 µg. of formaldehyde, equivalent to 0·5 µg. of glycine.

REAGENTS.
1. *Sodium tungstate*, 10% solution.

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* Abdehalden, E., and Schmidt, H., *Z. Physiol. Chem.*, 1911, **72**, 37; Virtanen, A., and Laine, T., *Skand. Arch. Physiol.*, 1938, **80**, 392.
† Alexander, B., Landwehr, G., and Seligman, A., *J. Biol. Chem.*, 1945, **160**, 51.

2. *Sulphuric acid*, 0·66N.

3. *Phosphate buffer*, pH 5·5.   Prepare by adding 3·5 g. K_3PO_4 to 100 ml. of 20% KH_2PO_4.

4. *Ninhydrin*, 1%.

5. *Chromotropic acid* (1:8-di-hydroxy-naphthalene-3:6-di-sulphonic acid), 5% *solution*.

METHOD. 1 volume of blood is treated with 7 volumes of water and 1 volume of sodium tungstate.   1 volume of 0·66N sulphuric acid is added, and after 15 minutes the mixture is filtered.   5 ml. of the filtrate are transferred to an all-glass distillation apparatus, 2 ml. of pH 5·5 buffer are added, followed by 1 ml. of ninhydrin solution and a glass bead.   The mixture is rapidly distilled into a test-tube graduated up to 10 ml. and about 7 ml. of the distillate are collected.

The distillation flask is then cooled to room temperature, 2 ml. of water are added to it and distillation is continued until the flask is just dry.   The neck of the condenser is heated gently to remove any drops which may have collected there.

The volume of distillate is then made up to 10 ml.   5 ml. of this are transferred to a tube which is cooled in an ice-bath, and 4 ml. of concentrated sulphuric acid are added.   The tube is then allowed to warm to room temperature, 0·1 ml. of chromotropic acid solution is added, and the tube is lightly stoppered and placed in a boiling water-bath for 30 minutes. After cooling, the colour is compared against standard glycine solutions which have been treated similarly.   If the method is to be used for urine, a fiftyfold dilution is made and it is necessary to run a blank with an aliquot of the diluted sample, replacing the ninhydrin solution with 1 ml. of water.

(h) Estimation of Tyrosine with Millon's Reagent*

PRINCIPLES. Mercuric nitrite and sulphuric acid gives a red colour with phenols and so this reaction may be applied to tyrosine.   If the acidity is between 3·5 and 7·5%, tryptophane, which also reacts, can be removed as a mercuric sulphate precipitate.

REAGENTS.

1. *Mercuric sulphate*, 15%.   Add 80 ml. of 20% sulphuric acid to 30 g. of mercuric sulphate and dissolve the slight precipitate which forms by addition of 31 ml. of water.   Finally make up to 200 ml. with 20% sulphuric acid and filter before use.

2. *Mercuric sulphate*, 1·5%.   Dilute 10 ml. of the above stock solution with 10 ml. of 60% sulphuric acid and make up to 100 ml. with water.

3. *Sulphuric acid*, 0·1N.

* Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.

4. *Sulphuric acid, 2N.*

5. *Sulphuric acid, 20%.*

6. *Sodium nitrite, 2% solution.*

METHOD. The sample, freed from tryptophane by precipitation with mercuric sulphate in $2N$ sulphuric acid, is transferred to a 100 ml. flask. Into a similar flask are put 5·0 ml. of (a 0·1%) tyrosine standard in $2N$ sulphuric acid. Each flask is then treated with the two mercuric sulphate solutions so as to contain 4·0 ml. of 15% mercuric sulphate, 12·0 ml. of 1·5% mercuric sulphate, and 12·0 ml. of $0·1N$ sulphuric acid. To each flask is then added sufficient 20% sulphuric acid to make the total acidity equivalent to 100 ml. of N acid. The flasks are then heated on a boiling water-bath for 15 minutes, cooled to room temperature, and 1·0 ml. of 2% sodium nitrite solution is added to each, with shaking. The colours, which tend to become turbid on standing, should be compared as quickly as possible.

(i) Estimation of Tryptophane with Ehrlich's Reagent and Nitrous Acid*

PRINCIPLES. Tryptophane gives with Ehrlich's reagent (*p*-dimethylamino-benzaldehyde) and sodium nitrite in sulphuric acid solution a blue colour which is admirably suitable for estimation in protein hydrolysates. Blue colours can also be obtained by the use of benzaldehyde† or glyoxylic acid.‡

REAGENTS.

1. *Sulphuric acid, 18·7N.*

2. *p-Dimethylaminobenzaldehyde, a 3% solution in 2N sulphuric acid.*

3. *Sodium nitrite* (freshly prepared). A 0·068% solution is prepared by diluting a Molar (6·9%) solution just before use.

METHOD. The tryptophane is obtained in neutral solution and 1 ml. is mixed with 7 ml. of $18·7N$ sulphuric acid and 2 ml. of *p*-dimethylamino-benzaldehyde solution. The mixture is cooled to 25° C. and kept in the dark at this temperature for 4 hours. To this solution is then added 0·1 ml. of the 0·068% solution of sodium nitrite and after mixing it is kept at room temperature, in the dark, for 60 minutes. When this time has elapsed the blue colour is measured by comparison against a similarly treated standard.

(k) Estimation of Histidine (in Urine)§

PRINCIPLES. If histidine is brominated in acid solution and then made alkaline, a blue coloration results.

* Spres, J., and Chambers, D., *Analyt. Chem.*, 1948, **20**, 30.
† Luscher, E., *Biochem. J.*, 1922, **16**, 556.
‡ Cary, C. A., *J. Biol. Chem.*, 1928, **78**, 377.
§ Langham, W. D., *J. Biol. Chem.*, 1941, **137**, 255.

REAGENTS.

1. *Histidine standard.* Dissolve 0·24 g. of histidine hydrochloride in 100 ml. of distilled water.

2. *Potassium permanganate, solid.*

3. *Acid-washed charcoal.*

4. *Sulphuric acid,* 10%.

5. *Bromine,* 1% *in carbon tetrachloride.*

6. *Phenol,* 0·5% *in water.*

7. *Sodium acetate, saturated solution.*

METHOD. 25 ml. of urine are treated with 5 ml. of 10% sulphuric acid and solid potassium permanganate (about 75 mg.) so that a purple colour persists for about 15 seconds. The mixture is then decolorised by the addition of 0·5 g. of acid-washed charcoal, and filtered twice through the same paper. 6·0 ml. of the colourless filtrate are transferred to each of two tubes (*a* and *b*) graduated at 10 ml. Into a third tube (*c*) are placed 1·0 ml. of histidine solution plus 5·0 ml. of 2·5% sulphuric acid.

To one of the tubes (*b*) containing filtrate is added 0·4 ml. of standard histidine solution. The three tubes are then connected by leads to a 1% solution of bromine in carbon tetrachloride, and bromine vapour is aspirated into them at a rate such that the brown colour is always present.

After aspiration for 20 minutes, 1·0 ml. of 0·5% phenol solution is added to each tube, followed by 1·0 ml. of saturated sodium acetate solution. After mixing, the tubes are immersed in a boiling water-bath for 1 minute, then cooled in an ice-bath, made up to 10 ml. with water, and compared in a colorimeter.

Throughout the whole of these operations care should be taken to avoid exposure of the solutions to light.

Since some colour is produced from contaminants such as urea, whilst others tend to suppress the reaction, it is essential to make a correction for this error. Now tube (*a*) contains the unknown quantity (*x*) of histidine, tube (*b*) contains (*x*+0·4 mg.), and tube (*c*) 1·0 mg.; but the observed concentrations in tubes (*a*) and (*b*) will both be lower than their true values, the apparent difference between tubes (*a*) and (*b*) thus corresponding to less than 0·4 mg. of histidine. In this way a correction factor may be arrived at.

(1) Estimation of Cysteine by *o*-Benzoquinone*

PRINCIPLES. Cysteine gives a red colour with a chloroform solution of *o*-benzoquinone. Cystine gives no colour under the same conditions. Reaction *e* (p. 348) can also be used, and in this case the colour is not

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* Hazeloop, E., (abstract in) *Chemie et Industrie*, 1935, **33**, 325.

discharged with sodium hydrosulphite ($Na_2S_2O_4$), as occurs with other amino-acids.*

REAGENTS.

1. *o-Benzoquinone*, 1% *in chloroform.*

2. *Artificial standard.* Prepared from 1·25 g. of cobalt acetate in 10 ml. of water. 1 ml. of 9% ferric chloride is added. The resulting colour approximates that from 2 mg. of cysteine.

METHOD. To 2 ml. of the solution containing cysteine, made acid to pH 1·0 with hydrochloric acid, are added 2 ml. of 1% o-benzoquinone solution. The mixture is well shaken for 5 minutes. The chloroform layer is allowed to separate, is removed, and dried over anhydrous sodium sulphate. The clear solution is then compared in a colorimeter or a photo-electric absorptiometer against a standard which has been treated similarly, or, if of the order of 2 mg., is compared against the artificial standard.

(m) Estimation of Creatine and Creatinine (in Urine)†

PRINCIPLES. Creatinine reacts with alkaline picrate solution to produce a red colour. Creatine is converted to the internal anhydride creatinine by heating with acids, and may then be measured in the same way.

REAGENTS.

1. *Picric acid, saturated aqueous solution.*

2. *Sodium hydroxide, 10% solution.*

METHOD.

(a) *Creatinine.* 2·0 ml. of urine are transferred to a 100 ml. flask and 20 ml. of picric acid solution, followed by 1·5 ml. of 10% sodium hydroxide, are added. The mixture is allowed to stand for 10 minutes and is then diluted to the mark and compared in a colorimeter against similarly treated standards.

Since there is no direct relationship between the concentration of creatinine and the colour measurable, the standard should not differ from the unknown by more than 10%. The best comparison is given if the sample contains about 1 mg. of creatinine, and the quantity of urine taken can be adjusted to this condition.. The better method is to read the colour in a photo-electric instrument previously calibrated against a series of standards which have been developed in the same way.

(b) *Creatine plus Creatinine.* 1·0 ml. of urine is pipetted into a 300 ml. flask and 20 ml. of picric acid solution are added. About 150 ml. of water are added and the mixture is boiled for 45 minutes under reflux, and then

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* Sullivan, M. X., and Hess, W. C., *J. Biol. Chem.*, 1937, **117**, 423.
† Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

reduced by boiling to about 20 ml. After cooling, 1·5 ml. of sodium hydroxide are added, the mixture is allowed to stand for 10 minutes, and the red colour is measured as before.

The difference between this figure and that given by creatinine gives the percentage of creatine.

(o) Estimation of Glucosamine Using p-Dimethylaminobenzaldehyde*

PRINCIPLES. Glucosamine acetate reacts with p-dimethylaminobenzaldehyde (Ehrlich's reagent) to give a red colour. The method is also applicable to mucin.

If the method is applied to urine, then a prior extraction of urobilinogen with ether should be made.

REAGENTS.

1. p-Dimethylaminobenzaldehyde. Recrystallise the reagent from alcohol and dissolve 0·8 g. in 300 ml. of alcohol to which 30 ml. of concentrated hydrochloric acid has been added.

2. Sodium methylate, 5% (freshly prepared).

3. Acetic anhydride.

4. Sodium hydroxide, 30%.

5. Natural standard. Dissolve 100 g. of glucosamine hydrochloride in 100 ml. of water saturated with chloroform. 1 ml. of this solution contains 1 mg. of glucosamine hydrochloride or 0·83 mg. of glucosamine.

6. Artificial standard. Dissolve 0·1 g. of phenol red in 28·2 ml. of 0·01N sodium hydroxide and make up to 500 ml. with water. Add 0·8 ml. of this solution to 100 ml. of borate buffer of pH 8·6 (prepared by mixing 50 ml. of 0·2M boric acid in 0·2M potassium chloride with 12 ml. of 0·2M sodium hydroxide and making up the solution to 200 ml.). 1 ml. corresponds to 1 mg. of glucosamine.

METHOD. Samples are suitably prepared and diluted so as to contain 1–5 mg. of glucosamine. Both the sample (a) and the standard solution (b) are evaporated to dryness on a water-bath in porcelain dishes. 1 ml. of sodium methylate solution is added to each sample, mixed thoroughly by rubbing with a glass rod, and the products are cooled and allowed to stand for 5 minutes. 0·3 ml. of acetic anhydride is then added, drop by drop, to each dish. After 5 minutes 1 ml. of water is added and the residues are transferred to filters. Each filter is washed with 1 ml. of water and then with 0·5 ml. of water and the washings are added to the filtrates. To each filtrate 1·0 ml. of alcohol and 0·5 ml. of 30% sodium hydroxide are added, and they are placed in a water-bath at 20° C. for 10 minutes. Heating is then made in a boiling water-bath for exactly 90 seconds and the tubes

* Kinji Kawabe, *J. Biochem. Japan*, 1934, **19**, 319-27.

are cooled in water at 20° C. for 10 minutes. 3·0 ml. of *p*-dimethylamino-benzaldehyde are then added, and, after standing for 5 minutes, the colours are compared.

(p) Estimation of Pyrrole*

PRINCIPLES. Pyrrole, which is separated from interfering substances by ether extraction, is transferred to glacial acetic acid solution and made to react with isatin.

REAGENTS.
1. *Sodium hydroxide*, 0·1*N*.
2. *Hydrochloric acid*, 6*N*.
3. *Isatin, a* 0·05% *solution in glacial acetic acid*.

METHOD. To a solution containing from 0·1–90 mg. pyrrole, 0·1*N* caustic soda is added until the *p*H of the solution is about 7·0. The solution is then extracted five times with 1/4th its volume of ether.

Into a litre Kjeldahl flask are placed 10 ml. of glacial acetic acid which are maintained on a water-bath at 50° C. The ethereal solution of the pyrrole is added, dropwise, with a current of air passing through so as to allow the ether to evaporate. The acid solution is then made up to 100 ml. and an aliquot is used for estimation. Care should be taken to avoid exceeding the temperature of 50° C., since there is possibility of transformation of pyrrole into resin at above this point.

An aliquot of the pyrrole solution is mixed with 2 ml. of 6*N* hydrochloric acid, made up to 10 ml. with glacial acetic acid, and 1 ml. of isatin solution is added. The mixture is then heated to boiling temperature for 5 minutes, cooled, and compared against standards.

(q) Estimation of Hydroxylamine by Benzoyl Chloride and Ferric Chloride†

PRINCIPLES. Hydroxylamine reacts with benzoyl chloride to give benzoyl hydroxamic acid. With ferric salt in neutral or slightly acid solution this reacts to give a reddish-violet colour.

REAGENTS.
1. *Benzoyl chloride, colourless*.
2. *Sodium acetate*, 2% *solution*.
3. *Ferric chloride*, 0·5% *in* 2% *hydrochloric acid by volume*.

METHOD. To 4·0 ml. of a sample which is neutral or slightly acid to phenolphthalein, and contains 0·8–3·5 mg. of hydroxylamine hydrochloride, are added 2 drops of colourless benzoyl chloride followed by 4·0 ml. of alcohol, and 2·0 ml. of sodium acetate solution. Shake for 30 seconds,

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* Fromm, F., *Mikrochem.*, 1935, **17**, 141.
† Pucher, G. W., and Day, H. A., *J. Amer. Chem. Soc.*, 1926, **48**, 672-6.

allow the mixture to stand for a few minutes, and then add 2·0 ml. of ferric chloride solution.   Dilute to 25 ml. and wait for 5 minutes before reading the colour.

The comparison is made with 4 ml. of a standard containing about 0·2 mg. of hydroxylamine per millilitre, which has been treated similarly.

(r) Estimation of Hexamethylenetetramine (in Urine) with Phloroglucinol*

PRINCIPLES. Hexamethylenetetramine (urotropine) is converted into formaldehyde by distillation at controlled pH.   Formaldehyde reacts with phloroglucinol to give a pink to red coloration.

REAGENTS.

1. *Phloroglucinol, a* 1% *solution in* 10% *sodium hydroxide.*

2. *Artificial standard.* A 0·025% solution of Congo red in water is prepared. This is matched against a solution of 1·7616 g. potassium bichromate and 11·5537 g. sulphuric acid (standardised by titration) diluted to 50 ml. This standard should match 2·5 ml. of standard Congo red diluted to 50 ml., and is equivalent to the colour given by formaldehyde in concentration 1 part in 100,000.

METHOD. A sample of urine is adjusted to pH 9·0 and distilled until its volume is reduced to about one-half.   A sample of the distillate (which should contain about 2·5 mg. of formaldehyde in 50 ml.) is taken, a few drops of 50% sodium hydroxide solution and 2 ml. of 1% phloroglucinol solution are added.   After mixing and allowing to stand for 3 minutes, the colour is compared with artificial standards.

If the original solution is strongly coloured, a yellow filter should be interposed before matching.

(s) Estimation of Sulphonamides (in Blood)†

PRINCIPLES. Sulphonamides, when diazotised and coupled with N(1-naphthyl)-ethylenediamine hydrochloride, produce a red colour suitable for estimation (compare p. 311).

Naturally sulphapyridine, sulphathiazole, and other secondary amines do not give similar depths of colour.‡

REAGENTS.

1. *Saponin,* 0·05% *solution.*

2. . *Trichloracetic acid,* 15% *solution.*

3. *Sodium nitrite,* 0·1% *solution, freshly prepared.*

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* Shohl, A. T., and Deming, C. L., *J. Urology*, 1920, **4**, 419-37.

† Bratton, A., Marshall, E., Babbitt, D., and Hendruckson, A., *J. Biol. Chem.*, 1939, **28**, 537.

‡ Allport, N. L., "Colorimetric Analysis" (London, 1945), p. 272.

4. *Ammonium sulphamate, 2% solution.*

5. *N(1-naphthyl)-ethylenediamine hydrochloride, 1% aqueous solution.*

METHOD. 2·0 ml. of oxalated blood are pipetted into 30 ml. of saponin solution. After a few minutes 8 ml. of trichloracetic acid solution are added, and the mixture is shaken thoroughly and filtered. 10 ml. of the filtrate are transferred to a test-tube and 1·0 ml. of sodium nitrite solution is added. After standing for 3 minutes, 1·0 ml. of ammonium sulphamate solution is added, and the mixture is shaken and allowed to stand for a further 10 minutes. At the end of this period 1·0 ml. of N(1-naphthyl)-ethylenediamine hydrochloride solution is added, and the resulting red colour is matched against standards. Conjugated sulphonamides are not estimated by this method unless a previous hydrolysis is carried out. This is best effected by taking 10 ml. of the trichloracetic filtrate, adding 0·5 ml. of 4N hydrochloric acid, and heating on a water-bath for 1 hour. After cooling the colour reaction is carried out as before. The method is most suitable for a range from 0·02–0·1 mg. of sulphanilamide.

9. VITAMINS, HORMONES, ENZYMES, ETC.

(a) Colorimetric Estimation of Vitamin A in Liver Oils*

PRINCIPLES. The estimation of vitamin A is dependent on the brilliant blue colour produced when the vitamin is treated with antimony trichloride in chloroform solution. Since the vitamin A resides in the unsaponifiable fraction of oils, this separation is made before the test is carried out. The colour reaction is of a transient nature and is modified by other substances present in the unsaponifiable fraction. For this reason it is considered that absorption at 325 mμ should be taken as due to vitamin A alone.†

REAGENTS.

1. *Antimony trichloride in chloroform.* Chloroform, containing 1% alcohol added as preservative, is washed three times with an equal volume of water and then dried over anhydrous potassium carbonate. The chloroform is poured off from the alkali, and while screened from light is distilled. The first 10% of the distillate is rejected. A quantity of antimony chloride is then washed with the chloroform until the washings are clear, and the antimony chloride is then dissolved in the chloroform to give a 22% w/v $SbCl_3$ solution. This is checked by iodine titration.

2. *Potassium hydroxide, 60% solution.*

3. *Ether, peroxide-free.*

* Allport, N., "Colorimetric Analysis" (Chapman and Hall, London, 1945), p. 352.
† See also Bowen, J. L., Gridgeman, N. T., and Longman, G. F., *Analyst*, 1946, **71**, 20; Mann, T. B., *Analyst*, 1943, **68**, 233.

METHOD. About 1 g. of the oil is mixed with 20 ml. of 95% alcohol, and 4·4 ml. of 60% potassium hydroxide are added. The mixture is boiled until a clear liquid results (usually about 5 minutes). The solution is then transferred to a separating funnel, the flask is rinsed with 40 ml. peroxide-free ether, and the ether solution is added to the funnel, the contents of which are then diluted with 60 ml. of water. A further 40 ml. of ether are added, the mixture is shaken, and the ether is allowed to separate. The aqueous layer is removed and extracted with two further 40 ml. portions of ether. The ether layers are mixed and washed four times with 50 ml. portions of water at 30° C. The ether layer is then dried over anhydrous sodium sulphate, transferred to a distilling flask, and the ether is removed in an atmosphere of nitrogen.

The unsaponifiable residue is then dissolved in chloroform, the inert atmosphere being maintained. 0·2 ml. of this solution is transferred to a 1 cm. glass cell and placed in the Lovibond tintometer. 2·0 ml. of the antimony trichloride reagent are added, and the blue colour which quickly develops is matched at its maximum intensity using blue, yellow, or red glasses.

This test is considered as an approximation, and it is repeated several times if necessary, diluting the chloroform solution so that the reading lies between 4·0 and 6·0 "blue units."

The vitamin A potency is expressed as the number of "Lovibond blue units" given by 0·04 g. of the original sample in 0·2 ml. of solvent. This result is converted to *International Units per Gram* by multiplying by 32.

(b) Colorimetric Estimation of Carotene*

PRINCIPLES. Carotene is extracted with acetone-petroleum mixture. Chlorophyll and flavones are removed by treatment with alkali, xanthophylls by methyl alcohol extraction, and carotene is finally compared against an artificial standard of potassium dichromate.†

REAGENTS.

1. *Acetone/light petroleum mixture.* 1 part of acetone to 3 parts of light petroleum of B.P. 40–60.

2. *Potassium hydroxide, a 30% solution in methyl alcohol.*

3. *Methyl alcohol, 90%.*

4. *Potassium dichromate, 0·025%.* This is considered as having the same colour intensity as 0·158 mg. of β carotene in 100 ml. of light petroleum.

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* Seaber, W., *Analyst*, 1940, **65**, 266.

† Goodwin, T. W., and Morton, R. A. (*Analyst*, 1946, **71**, 15) describe a method for determining Carotene and Vitamin A in butter and margarine. The unsaponifiable fraction is dissolved in hexane and the xanthophylls are removed by shaking with 90% methyl alcohol. The extinction coefficient of the yellow solution is then measured at 450 mμ using the Hilger-Nutting Spectrophotometer.

METHOD. 5 g. of material are ground in a mortar with about 100 g. of silver sand of No. 60 mesh. The mixture is then transferred to a very fine extraction thimble, and is extracted with 60 ml. of acetone/light petroleum mixture until colour is no longer removed. The extract is transferred to a separating funnel and 5 ml. of freshly prepared 30% potassium hydroxide in methyl alcohol are added. After shaking for 2 minutes, 200 ml. of water are introduced, and the funnel is inverted two or three times. After settling, the aqueous layer is removed and the petroleum layer is washed with a further 200 ml. portion of water. The light petroleum fraction is then shaken once with 30 ml. and twice with 15 ml. portions of 90% methyl alcohol. After settling, the light petroleum fraction is clarified by shaking with a little anhydrous sodium sulphate, completed to 50 ml., and compared in a colorimeter against the dichromate artificial standard.

(c) Estimation of Vitamin B_1 (Aneurin)*

PRINCIPLES. Aneurin is separated by adsorption on zeolite, oxidised to thiochrome with alkaline ferricyanide, the blue fluorescence of which is measured in an instrument using mercury vapour as the light source.

The daily excretion of vitamin B is in the order of 70 μg., and the method will measure 0·1 μg.

REAGENTS.

1. *Acetic acid*, 0·2N.
2. *Zeolite* (Decalso brand).
3. *Acetic acid*, 1%.
4. *Alcohol*, 95%.
5. *Potassium chloride*, 25% *solution*.
6. *Potassium ferricyanide*, 0·5% *solution*.
7. *Caustic soda*, 2·5% *solution*.
8. *Iso-butyl alcohol*.

METHOD (*for estimation in urine*). From a 24-hour specimen of urine containing 0·1% of acetic acid as a preservative, 12 ml. are transferred to a glass-stoppered centrifuge tube and shaken with 10 ml. of *iso*-butyl alcohol in order to remove fluorescent substances which would interfere with the subsequent estimation. After centrifuging, the alcohol layer is completely removed, and 9 ml. of the urine layer are diluted with 10 ml. of water. Using 0·2N acetic acid, the reaction mixture is adjusted to pH 4·2, using bromophenol blue as an external indicator. The solution is then poured into a narrow Gooch crucible which contains about 0·2 g. of Decalso zeolite, which has been twice washed with 1% acetic acid followed by two washings with 95% alcohol and one washing with acetone.

* Egaña, E., and Meiklejohn, A. P., *J. Biol. Chem.*, 1941, **141**, 859.

The urine solution is drawn through by gentle suction and the zeolite is twice washed with 10 ml. of 1% acetic acid.   The zeolite is transferred to a centrifuge tube, the sides of the Gooch are washed down with 2 ml. of 25% potassium chloride, and the washings are added to the centrifuge tube. After shaking thoroughly, the tube is centrifuged, the supernatant liquid is removed, and potassium ferricyanide is added drop by drop to it until the solution is just yellow.   This usually takes about 0·3 ml.   Finally, 1 ml. of 20% caustic soda is added.

4·9 ml. of *iso*-butyl alcohol are pipetted into the tube and the mixture is thoroughly shaken for 1 minute.   Separation is made by centrifuging. The *iso*-butyl alcohol layer is filtered into a dry test-tube and transferred to a photo-electric instrument with a mercury-vapour lamp as the light source.   The intensity of the blue fluorescence is then measured and compared against standard solutions of aneurin which have been oxidised in the same way.

In order to eliminate errors due to substances with fluorescent tints other than the purplish-blue due to thiochrome, the light is filtered using Wood's glass in conjunction with a Wratten 18A filter.

(d) Colorimetric Estimation of Nicotinic Acid and Nicotinamide*

PRINCIPLES. Nicotinamide is hydrolysed with caustic soda to nicotinic acid.   Treatment with cyanogen bromide produces a quarternary nitrogen compound which, on coupling with an aromatic amine, produces a coloured dianil derivative.

The daily excretion of nicotinic acid+nicotinamides is from 0·5–2·5 mg., and the method will measure 1 µg. of nicotinic acid.

REAGENTS.

1. *Caustic soda*, 40%.

2. *Iso-butyl alcohol.*

3. *Potassium permanganate*, 4% *solution.*

4. *Hydrochloric acid*, 10%.

5. *Cyanogen bromide solution.* Titrate bromine water with 10% sodium cyanide solution until just colourless.

6. *p-Aminoacetophenone*, 10% *solution in* 96% *alcohol.*

METHOD (*as applied to urine*). To 20 ml. of urine are added 2 ml. of 40% caustic soda, and the mixture is heated on a water-bath for 45 minutes. After cooling, the *p*H of the solution is adjusted to 2·5 with hydrochloric acid and the solution is made up to 25 ml. with water.   12·5 ml. of *iso*-butyl alcohol are pipetted on to the solution, which is then shaken for 2 minutes. 20 ml. of the aqueous layer are transferred to a beaker and placed on a boiling

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* Wang, Y. L., and Kodicek, E., *Biochem. J.*, 1943, **37**, 530.

water-bath in order to evaporate off the bulk of the dissolved alcohol. 15 ml. of water are added, the temperature of the mixture is brought to 40° C., 0·3 ml. of hydrochloric acid is added, and 4% potassium permanganate solution is run in drop by drop until a pink colour persists for at least 30 seconds. The solution is then cooled and allowed to stand aside for 15 minutes to destroy the permanganate colour. The solution is then adjusted to pH 6·5 with 40% caustic soda and made up to 50 ml. Any precipitate is removed by centrifuging.

Into each of four tubes with a 15 ml. graduation are placed 10 ml. of the prepared urine solution. Into the first (a) is put 0·6 ml. of 10% hydrochloric acid, and into (c) and (d) is placed 0·5 ml. of a standard acid solution containing 10 µg. of nicotinic acid. To each tube are then added 2·0 ml. of cyanogen bromide solution. The tubes are then placed in a water-bath at 56° C. for 4 minutes and·then are allowed to cool for 5 minutes in the absence of light. At the end of this period 1 ml. of p-aminoacetophenone solution is added to each tube, and to (b), (c), and (d) 0·6 ml. of 10% hydrochloric acid also. After standing for a further period of 5 minutes in the dark, the colours in the tubes are measured in a photo-electric absorptiometer. The reading given by (a) will represent a blank, (b) the urine to be estimated, and that of (c) and (d) will represent the urine concentration+standard nicotinic solution. From these figures the urine concentration may be calculated.

(e) Colorimetric Estimation of Vitamin C (Ascorbic Acid)*

PRINCIPLES. The vitamin is extracted with acetic acid and treated with a special silico-molybdate reagent which becomes reduced to molybdenum blue. The method is particularly suitable for fresh vegetables, etc., where other reducing substances are present in low concentration. It is not recommended for urine in preference to the indophenol titration (see p. 177), since the results tend to be too high due to the presence of other reducing substances. The reagent is more readily reduced with ferrous and stannous ions, but less so with sulphydryl groups, sulphites, and thiosulphates.

The method will measure 0·01 mg. of the vitamin in 50 ml. of solution.

REAGENTS.

1. *Acetic acid,* 5% *solution.*

2. *Silico-molybdate reagent.* Dissolve 2 g. of ammonium molybdate in 50 ml. of water at 55° C. Add 10 ml. of 1% sodium silicate ($9H_2O$) solution, 5 ml. of glacial acetic acid, and complete to 100 ml. with water. Leave over-night.

METHOD. 5 g. of the sample are ground with sand and 5% acetic acid solution in a mortar, and are filtered. The sand is washed with acetic acid

* Isaacs, M. L., *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14,** 948.

and the combined solutions are mixed and made up to a given volume. An aliquot portion of this, containing not more than 0·5 mg. of ascorbic acid, is transferred to a 50 ml. flask and made up to about 25 ml. with water. The reagent is then added in the proportion of 1·0 ml. for each 0·1 mg. of vitamin, and the volume is made up to 50 ml. with water. Colour development is almost immediate, and the product is stable for at least 48 hours. Colour measurement is made preferably in a photo-electric absorptiometer using a yellow filter. If comparison is made in a colorimeter against standards, a yellow filter should be fitted in the eyepiece to obviate the residual colour of the reagent.

(f) Colorimetric Estimation of Vitamin E*

PRINCIPLES. α-tocopherol is rapidly oxidised by ferric chloride provided that the ferrous ions are progressively removed. In the presence of αα-dipyridyl, ferrous ions produce a red colour which is thus proportional to the amount of vitamin present.

REAGENTS.

1. *Potassium hydroxide, 0·2N.*

2. *Formaldehyde, 38%.*

3. *Ether, peroxide-free.*

4. *Potassium hydroxide, 2%.*

5. *Sulphuric acid, 1%.*

6. *Ferric chloride, a 0·2% solution of hydrated ferric chloride in absolute alcohol.*

7. *αα-dipyridyl, a 0·5% solution in absolute alcohol.*

METHOD (*as applied to blood*). 10 ml. of blood are transferred to a 250 ml. separator. To this are added, successively, with shaking between additions, 5 ml. of 0·2N potassium hydroxide, 15 ml. of neutralised formalin solution, and 15 ml. of 95% alcohol. The mixture is extracted three times with 50 ml. portions of peroxide-free ether, using 10 ml. of alcohol each time to avoid emulsion formation. The combined extracts are washed with 2% potassium hydroxide, 25 ml. of 1% sulphuric acid, and finally three times with water. The ether extract is dried over anhydrous sodium sulphate and filtered directly into a distilling flask. 10 ml. of benzene are added, a current of carbon dioxide is passed through the apparatus, and the solvent is removed by gentle distillation. The residue is dissolved in 5 ml. of benzene and then is passed through a column of fuller's earth 3 cm. long and 1 cm. diameter. The earth is washed five times with 5 ml. of benzene. The total benzene eluate and washings are transferred to a distillation flask and the solvent is removed in a current of carbon dioxide. The residue in the flask is then dissolved in 5 ml. of

* Emmerie, A., and Engel, C., *Rec. Trav. Chim.*, 1939, **58**, 895.

a mixture of 1 ml. of ferric chloride in alcohol, 1 ml. of αα-dipyridyl solution, and 5 ml. of benzene. The mixture is then allowed to stand for 10 minutes away from light and the red colour is measured immediately, preferably in a photometer. Comparison against standard tocopherol solutions should be carried out as rapidly as possible, but substitute standards of ferrous iron may be used.

(g) Colorimetric Estimation of Adrenalin (in Blood)*

PRINCIPLES. Adrenalin is adsorbed on specially prepared aluminium hydroxide, and the eluate is treated with arseno-molybdic acid to produce a blue colour. The method will detect 0·2 μg. of adrenalin in venous blood.

REAGENTS.

1. *Arseno-molybdic acid solution.* Dissolve 60 g. of sodium molybdate and 10 g. of sodium arsenate in 200 ml. of water. After filtration add 5 ml. of bromine water, dilute the solution to 500 ml. with water, and finally add 40 ml. of concentrated sulphuric acid.

2. *Sulphuric acid.* Equal volumes of concentrated sulphuric acid and water.

3. *Sodium sulphite solution.* Dissolve 10 g. of sodium sulphite in 50 ml. of water. This solution should be freshly prepared. Immediately prior to use add 2 ml. of it to 14 ml. of 50% sulphuric acid.

4. *Phenolphthalein solution.* Dissolve 0·1 g. of phenolphthalein in 100 ml. of 0·01N sodium hydroxide.

5. *Aluminium hydroxide suspension.* Dissolve 25 g. of potassium alum in 200 ml. of hot water. After cooling add slowly, with stirring, 25 ml. of 25% sodium hydroxide solution. Filter the precipitate off, wash thoroughly, and then suspend in 100 ml. of water.

6. *Standard adrenalin solution.* Dissolve 100 mg. of adrenalin in 100 ml. of 0·01N sulphuric acid. This solution is diluted 1,000 times prior to use, 0·01N sulphuric acid acting as a diluent.

METHOD. 5·0 ml. of blood are run into 20 ml. of 10% trichloracetic acid, and after mixing the precipitate is separated by centrifuging and the clear supernatant fluid is passed into a test-tube. 1·0 ml. of the filtrate is transferred to another centrifuge tube, and 1·0 ml. of 0·04N sulphuric acid and 2 drops of phenolphthalein solution are added. The mixture is titrated with sodium hydroxide to pH 8·0 (just pink) and to this are then added 2·0 ml. of aluminium hydroxide suspension. The mixture is again adjusted to pH 8·0, thoroughly shaken, and then allowed to stand for 5 minutes. It is centrifuged at high speed for 5 minutes. After removal of the supernatant fluid and draining the residual liquid by inversion, the precipitate

* Shaw, F., *Biochem. J.*, 1938, **32**, 19; Bloor, W., and Bullen, S., *J. Biol. Chem.*, 1941, **138**, 727.

is dissolved in 2·0 ml. of arseno-molybdic acid solution and the tube is immersed in a boiling water-bath for 5 minutes. 3·0 ml. of sulphuric acid reagent are next added; the mixture is heated at boiling temperature for a further 10 minutes and finally cooled in ice-water for 15 minutes. After dilution to 40 ml. the colour is matched against a standard containing about 0·5 μg. of adrenalin that has been subjected to the same procedure.

(h) Colorimetric Estimation of Androgens* (17-ketosteroids)

PRINCIPLES. Urine is refluxed with hydrochloric acid to hydrolyse conjugated bodies, and aromatic substances are extracted with benzene. Phenolic substances are removed by alkali treatment and the residue is estimated for methylene-ketone ($-CO-CH_2-$) groups by the Zimmermann reaction with m-dinitrobenzene.

REAGENTS.

1. *Sodium carbonate, saturated solution.*

2. *Sodium hydroxide, 2N.*

3. *Absolute alcohol* (aldehyde-free). Aldehydes are removed by distillation over m-phenylenediamine.

4. *m-Dinitrobenzene, 2% in aldehyde-free alcohol.* The m-dinitrobenzene must be freed from thiophene derivatives by treatment with alkali and repeated crystallisation.

5. *Potassium hydroxide, 5N in absolute alcohol, freshly prepared.*

METHOD. The urine specimen (usually a 3-day sample) is adjusted to pH 1·0, using methyl violet indicator. The urine is then made more strongly acid by addition of 20 ml. hydrochloric acid per litre in excess. The mixture is then treated with 125 ml. of carbon tetrachloride per litre, and refluxed on a water-bath for 2 hours. After cooling, the carbon tretrachloride layer is removed by suction to a flask attached to a filter pump. The refluxing is then continued for a further 2 hour period, using fresh carbon tetrachloride, and this process is repeated a third time. The carbon tetrachloride extracts are united and distilled to dryness on a water-bath. The residue is then taken up in benzene using 100 ml. of benzene per litre of original urine. The solution is then freed from acid by washing twice with a quarter of its volume of saturated sodium bicarbonate solution. It is then freed from phenols by extracting five times with a fifth of its volume of 2N caustic soda, and finally is washed with water.

The benzene solution is then evaporated to dryness. The residue is extracted five times with 5 ml. of redistilled ether, and the extract is filtered through a sintered glass filter and evaporated to dryness on a water-bath in a stream of nitrogen. This residue is taken up in aldehyde-free absolute alcohol, so that each millilitre of solution is equivalent to 100 ml. of the

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* Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, 1938, **32**, 1312.

original urine. 0·1 ml. of the alcoholic solution is transferred to a dry test-tube and 0·1 ml. of alcohol and 0·2 ml. of *m*-dinitrobenzene solution are added. After mixing, 0·2 ml. of 5*N* alcoholic potassium hydroxide is added, and the tube is placed for 45 minutes in a water-bath at 25° C. excluded from light.

A blank is carried out at the same time, using 0·1 ml. of the extract but replacing the dinitrobenzene solution by alcohol. At the end of the development period the solutions are diluted to 10 ml. with alcohol and the red colour is measured in a photo-electric absorptiometer which is calibrated using standard solutions of androsterone.

(i) Colorimetric Estimation of Thyroxine*

PRINCIPLES. Thyroxine gives with diazobenzene-sulphonic acid and alkali a red coloration, sensitive to 10µg./5 ml.

REAGENTS.

1. *Sodium hydroxide.* (i) 10% in water; (ii) 3*N*.
2. *A diazotised solution of sulphanilic acid.* Approximately *M*/10.

METHOD. To 5 ml. of the thyroxine solution are added 4 ml. of 10% sodium hydroxide, and the mixture is cooled to 0° C. To it are then added 4 ml. of diazobenzene-sulphonic acid solution, also at 0° C., and 3 minutes are allowed to elapse for the completion of the reaction. 2 ml. of 3*N* sodium hydroxide are then added, the tube is allowed to stand at room temperature, and the red colour is measured after a further 5, but before 7, minutes, preferably in a photometer.

(k) Colorimetric Estimation of Cholesterol†

Example (i): *Benzoyl Chloride Method as Applied to Blood Plasma.*

PRINCIPLES. The most commonly used colour reaction for cholesterol is that originally due to Liebermann and Burchard [Example (ii)], and involves the production of a green colour when a chloroform solution of cholesterol is treated with acetic anhydride and sulphuric acid. Methods based on this principle are only approximate, since many substances besides cholesterol modify the colour reaction. Alternatively the colour reaction of *o*-nitrobenzoyl chloride and the separated digitonide may be used.

REAGENTS.

1. *Acetone/alcohol.* Mix equal volumes.
2. *Sodium ethylate,* 20% *in alcohol.*
3. *Light petroleum.*
4. *Digitonin,* 1% *solution in* 50% *alcohol.*

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* Moser, H., *Experientia,* 1947, **3**, 119.
† Obermer, E., and Milton, R., *Biochem. J.,* 1933, **27**, 345.

5. *Aluminium chloride, 4% solution.*

6. *Hydrochloric acid* (1 : 3).

7. *Zinc chloride, 3·5% in glacial acetic acid.* Weigh about 10 g. of stick zinc chloride into a hard glass flask. Heat the flask over a free flame until all moisture and fumes are driven off and a clear melt is obtained, and rotate the flask so that the melt distributes itself evenly over the surface of the flask. Allow this to cool down to below 100°. Then add glacial acetic acid and heat the flask gently until solution is obtained, and continue the addition until the solution is 3·5% with respect to zinc chloride. This solution must be kept in a stoppered bottle, observing precautions to avoid absorption of moisture. Even in low concentration the solution is so hygroscopic that standing in an open basin for a period of 1 hour causes 50% loss in its colour-producing power with regard to the reaction. Hence the stopper should not be left out of the bottle for a second longer than is necessary.

8. *o-Nitrobenzoyl chloride, 10% solution in glacial acetic acid.* Prepare this solution by melting solid acid chloride and diluting the liquid down with glacial acetic acid. After 3 or 4 days the acid chloride may have hydrolysed to the acid. A deterioration of this type is not permissible to any large extent, since a reduction in the concentration of the acid chloride causes a slowing down of the colour reaction.

METHOD.

(a) *Preparation of Alcohol/Acetone Extract.* About 15 ml. of alcohol/acetone (equal volumes) are introduced into a 25 ml. graduated flask. 1·25 ml. of plasma are run in from a pipette, drop by drop, with shaking. The flask is heated to boiling in a water-bath, with constant shaking, maintained at 100° for 3 minutes, and then is removed and allowed to stand for 15 minutes, made up to the mark, shaken vigorously, and then the contents are filtered. It is advisable *not* to use a fluted filter-paper; this does not accelerate filtration and causes a considerable loss in the volume of the filtrate.

(b) *Estimation of Free Cholesterol.* 10 ml. of alcohol/acetone extract are evaporated to dryness with 0·5 ml. of 1% digitonin solution. The last traces of alcohol are removed on the water-bath. 2 ml. of water are added to the flask, the contents are raised to boiling, and then the colloidal solution which forms is broken up by the addition of 4 ml. of acetone. The contents of the flask are transferred to a centrifuge tube, 1 drop of aluminium chloride solution is added, and after mixing, 1 drop of ammonia is used to precipitate aluminium hydroxide, which helps the digitonide precipitate to pack. The tube is then centrifuged at high speed for about 1 minute. The supernatant fluid is decanted off completely. The precipitate is dissolved in 1 drop of 30% hydrochloric acid. The original precipitation flask is washed out with a further 3 ml. of acetone, using a rod to aid removal of residual

digitonide from its sides. This wash fluid is then added to the centrifuge tube, the contents are mixed, and the whole is again centrifuged for about 5 minutes. The supernatant fluid is decanted off and the precipitate is washed again with 2 ml. of acetone.

Finally the precipitate is washed with 3 ml. of ether, centrifuged, decanted, and the residual ether is allowed to evaporate spontaneously. 3 ml. of zinc chloride/glacial acetic acid solution and then 2 ml. of acid chloride are added to the precipitate. The tube is covered and kept for 50 minutes in an oil-bath maintained at 100°. At the end of this time the tube is allowed to cool, and is diluted with an equal quantity of zinc chloride in glacial acetic acid.

The colorimetric reading is then taken.

(c) *Estimation of Total Cholesterol.* 5 ml. of alcohol/acetone extract are refluxed for 30 minutes with 0·5 ml. of sodium ethylate. At the end of this time the bulk of the alcohol is boiled off and about 15 ml. of petroleum ether are added. 2 ml. of water are then added, and the contents are thoroughly shaken. After settling, the petroleum ether layer is poured off through a filter-paper into a dry flask. The residue is washed twice more with 10 ml. of petroleum ether. To the combined petroleum ether extract is added 0·5 ml. of digitonin solution, and the bulk of petroleum ether is distilled off. 10 ml. of alcohol/acetone are then added, and the contents of the flask are evaporated to dryness on the water-bath. After this the procedure for free cholesterol estimation (as above) is continued with.

The colours produced in each case are compared against standard cholesterol solutions submitted to the same procedure.

Example (ii): *Liebermann-Burchard Reaction Applied to Blood Plasma.*[*]

REAGENTS.

1. *Alcohol/acetone* (1 : 1).

2. *Digitonin.* Dissolve 1 g. in 1 l. of water and filter. Concentrate to 500 ml. under reduced pressure and again filter.

3. *Potassium hydroxide, 50% solution.*

4. *Hydrochloric acid, 15%.*

5. *Acetic anhydride.*

METHOD.

(a) *Preparation of Extract.* 3 ml. of alcohol/acetone are pipetted into a 5 ml. flask, 0·2 ml. of serum is added, and, after mixing, the flask is placed in a boiling water-bath until the contents are just boiling. The flask is then cooled, the volume made up to 5 ml., thoroughly shaken, and filtered.

(b) *Estimation of Free Cholesterol.* 2·0 ml. of the filtrate are transferred to a centrifuge tube, 1 ml. of digitonin is added, and the mixture stirred

[*] Schoenheimer, R., and Sperry, W., *J. Biol. Chem.*, 1934, **106**, 745.

with a glass rod, covered, and allowed to stand over-night. The contents are again stirred and centrifuged. The precipitate is then washed twice with 2 ml. of acetone/alcohol, care being taken to wash down the sides of the tube each time. It is finally washed with ether and allowed to dry off.

Total Cholesterol. 1 ml. of filtrate is transferred to a centrifuge tube and 1 drop of 50% caustic potash is added. The tube is covered and warmed to 40° C. for 30 minutes. After cooling, 1 ml. of alcohol/acetone is added and the solution is titrated with 15% hydrochloric acid to a phenolphthalein end-point. It is essential that the solution be neutral since excess of acid inhibits digitonide formation and alkali changes cholesterol to some state which does not form a digitonide. 1·0 ml. of digitonin solution is then added and the washing and separation are carried out for free cholesterol.

(c) *Colour Reaction.* In each case the digitonide precipitate is dissolved in 1·0 ml. of acetic acid, warming if necessary to 60° C. to produce solution. After cooling to 25° C., 2·0 ml. of acetic anhydride and 0·10 ml. of sulphuric acid are added. The green colour is then matched against cholesterol standards after 27–37 minutes development time according to the room temperature.

(l) Colorimetric Estimation of Thymonucleic Acid*

Example (i): *Use of Schiff's Reagent.*

PRINCIPLES. If thymonucleic acid is hydrolysed, the resulting product behaves as an aldehyde and produces a violet colour with Schiff's fuchsine reagent. The pH of the solution, the time of hydrolysis, and the amount of reagent used, should be rigidly controlled. The optimum range for the method is from 0·5–2 mg. in 2 ml. of sample.

REAGENTS.

1. *Hydrochloric acid*, 0·05N.

2. pH 2·0 *buffer.* Dissolve 21·008 g. of citric acid in 200 ml. of N sodium hydroxide and complete to 1 l. with water. Mix 30·5 ml. of this solution with 65 ml. of 0·1N hydrochloric acid.

3. 2 : 4-*Dinitrophenol*, 0·05% *solution.*

4. *Fuchsine reagent.* Dissolve 1 g. of fuschine and 3 g. of sodium bisulphite in 40 ml. of N hydrochloric acid and allow the solution to stand until it turns to a faint straw colour.

METHOD. 2 ml. of the sample are treated with 8 ml. of 0·05N hydrochloric acid, heated on a boiling water-bath for 3 minutes, and cooled rapidly under running water. 2 drops of dinitrophenol indicator are added and the solution is titrated with 0·1N sodium hydroxide until the faint yellow colour appears. An equal volume of pH 2·0 buffer is added and the solution is

* Widstrom, G., *Biochem. Z.*, 1928, **199**, 298.

completed to 10 ml. with water. To this solution are then added 3·0 ml. of a mixture of 1 part of fuchsine reagent and 2 parts of buffer, and the colour is compared with a similarly treated standard.

Example (ii): *Use of Diphenylamine.**

PRINCIPLES. After hydrolysis the carbohydrates originally bound to the purine bases in the thymonucleic acid molecule will give a blue colour with a reagent consisting of diphenylamine in 1 part sulphuric acid and 39 parts glacial acetic acid. The colour reaction is due to the thyminose content. Salts of weak acids and protein decomposition products interfere by acting as buffers. Other carbohydrates do not interfere provided that the original conditions are adhered to.

The optimum range for the method is from 0·05–0·1% of thymonucleic acid.

REAGENT.
Diphenylamine reagent. 11 ml. of concentrated sulphuric acid are added to 400 ml. of a 1% diphenylamine solution made up in glacial acetic acid.

METHOD. The sample, which is freed from interfering substances by precipitation with alcohol and solution in water, is mixed with twice its volume of diphenylamine reagent. A standard is treated similarly. Both are heated in a boiling water-bath for 3 minutes, cooled in running water, and allowed to stand for a few hours before comparison is made. Initial yellow colours which may interfere are obviated if an appropriate yellow screen is fitted into the eyepiece of the measuring instrument.

(m) Colorimetric Estimation of Indole and Skatole as the Xanthyl Compounds†

PRINCIPLES. On treatment with xanthydrol, indole and skatole form dixanthylindole and dixanthylskatole, which when dissolved in glacial acetic acid and treated with trichloracetic acid give a reddish-blue colour.

REAGENTS.
1. *Xanthydrol reagent.* Dissolve 0·025 g. of xanthydrol in 10 ml. of glacial acetic acid.
2. *Trichloracetic acid,* 40%.
3. *Standard.* Mix 200 ml. of water and 25 ml. of 5% copper sulphate pentahydrate. Add 25 ml. of 6% sodium tungstate solution, mix, and filter. Transfer 2 mg. of indole to a 200 ml. flask and dilute to volume with the above solution. A similar standard can be prepared from skatole.

METHOD. Indole and skatole are extracted with ether. At the same time a suitable amount of standard (0·1 ml. usually) is extracted with 30 ml. of ether and treated similarly throughout. To both ether solutions is added

* Dische, Z., *Mikrochem.*, 1930, **8**, 4-32.
† Zappacosta, Mario, *Diag. tech. lab. Napoli. Riv. mensile*, 1935, **6**, 870.

1 ml. of xanthydrol reagent. They are then heated on a water-bath until the ether has evaporated. 0·25 ml. of 40% trichloracetic acid is added and the heating is continued for 5 minutes longer. 1·5 ml. of glacial acetic acid are then added and the mixture is transferred to a 10 ml. cylinder. The solutions are diluted to 2·5 ml. with acid and compared by the balancing method (p. 228).

(n) Colorimetric Estimation of Indoxyl (in Urine)*

PRINCIPLES. Indoxyl sulphate with thymol and ethyl trichloroacetate produces a red colour suitable for estimation.

REAGENTS.

1. *Potassium persulphate*, 1%.
2. *Thymol*, 1% *solution in* 95% *ethyl alcohol*.
3. *Trichloroacetic acid*. Dissolve 10 g. in 400 ml. of 50% hydrochloric acid.

METHOD. 1 ml. of filtered urine is diluted to 5 ml. with distilled water. 1·0 ml. of this solution is pipetted into a centrifuge tube with a graduation at 2 ml. To this is then added 0·1 ml. of persulphate solution followed by 0·25 ml. of thymol solution. After shaking, 2·5 ml. of trichloroacetic acid solution are then added and the tube is placed in a boiling water-bath for 5 minutes. The contents of the tube are cooled and centrifuged, and the watery layer is removed by means of an upturned capillary pipette (see p. 32). The ethyl trichloroacetic layer containing the pigment is diluted to the 2 ml. mark with glacial acetic acid and the red colour is measured in a micro-colorimeter. It has been found that cobalt sulphate is a suitable colour standard for indoxyl. Under the conditions for the above method, 0·75% $CoSO_4,7H_2O$ is equivalent to 0·0042 mg. of indoxyl in 2 ml. of solution.

(o) Colorimetric Estimation of Haemoglobin† (the Alkaline Haematin Method)

PRINCIPLES. The most generally favoured method of estimating haemoglobin depends upon conversion to alkaline haematin by treatment with caustic soda. Comparison is best made against standards which are prepared from pure haemin of standardised iron content.

REAGENTS.

1. *Sodium hydroxide*, 0·1N.

2. *Haemin standard*. 79·4 mg. of pure haemin containing 8·57% Fe are dissolved in 1 l. of 0·1N sodium hydroxide. This is equivalent to blood containing 15·6 g. per 100 ml., which is considered to be equal to 100% on the Haldane scale.

METHOD. 0·05 mg. of blood is pipetted into 5 ml. of 0·1N sodium hydroxide and heated in a boiling water-bath for 5 minutes. After cooling the

* Sharlit, H., *J. Biol. Chem.*, 1932, **99**, 537.

† Clegg, J., and King, E., *Brit. Med. J.*, 1942, **2**, 329.

brownish-red colour is compared against 5 ml. of the standard haemin solution which has been heated in the same way.

(p) Colorimetric Estimation of Bilirubin*

PRINCIPLES. The diazonium compound of sulphanilic acid reacts with bilirubin to produce a reddish-purple colour.

REAGENTS.

1. *Sulphanilic acid solution.* 1 g. in 250 ml. N hydrochloric acid diluted to 1 l. with water.

2. *Sodium nitrite, 0·5% solution.*

3. *Diazo reagent.* 10 ml. of sulphanilic acid solution are mixed with 0·3 ml. of sodium nitrite solution immediately prior to use.

4. *Ammonium sulphate, saturated solution.*

5. *Methyl red artificial standard.* 1 ml. of 0·29% solution of methyl red in glacial acetic acid is diluted to 5 ml. of glacial acetic acid and mixed with 14·4 g. of sodium acetate. Dilute to 1 l. This solution of pH 4·63 is equivalent to the colour given by 0·1 mg. of bilirubin in 25 ml.

METHOD. 1·0 ml. of plasma is mixed with 0·5 ml. of diazo reagent, 0·5 ml. of saturated ammonium sulphate, and 3 ml. of absolute alcohol. The mixture is thoroughly shaken, allowed to stand for a few minutes, and then filtered. The colour of the filtrate is then matched against standards.

With normal protein content the dilution of the plasma is approximately 1 in 4, so that the bilirubin can be taken as being that contained in 0·25 ml. of plasma.

Notes on Colorimetric Estimations of Enzymes

1. *Diastase*†

PRINCIPLES. Diastatic activity is determined by the action of an enzyme preparation on a starch paste substrate, the subsequent products being assessed on the basis of their copper-reducing power.

REAGENTS.

1. *Starch paste.* Suspend 100 g. of rice starch in 1 l. of 0·01N hydrochloric acid. After frequent agitation during an hour, allow the mixture to sediment and pour away the supernatant fluid. Treat the residue with 0·05%

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* King, E., Hazelwood, G., and Delory, G., *Lancet*, 1937, I, 886.
† Somogyi, M., *J. Biol. Chem.*, 1938, **125**, 399. Andersch, M. (*J. Biol. Chem.*, 1946, **166**, 705), gives an alternative method, using as the substrate amylase adsorbed on cotton-wool from potato starch. This may be dried, and it acts as a stable substrate which gives a clear solution. For the estimation a sample of the amylase-cotton is shaken with a buffered solution and the enzyme solution is added. After incubation, proteins, etc., are removed by precipitation with zinc sulphate and barium hydroxide and the reducing substances are measured by the method of Somogyi (*J. Biol. Chem.*, 1945, **160**, 61), using the colour reagent of Nelson (*J. Biol. Chem.*, 1944, **153**, 375). (See p. 333).

sodium chloride solution, stir well, and allow to sediment. Remove the supernatant fluid and wash the sediment once more with sodium chloride solution. Finally centrifuge and spread the washed starch out on a plate to dry in the air. Grind 15 g. of this in a mortar with 50 ml. of water, and pour the mixture slowly into 950 ml. of water. After boiling for 1 minute, heat it on a boiling water-bath for 30 minutes. This solution will keep for some time but should be renewed when mould appears.

2. *Acid sodium chloride solution.* Dissolve 10 g. of sodium chloride in water, add 3 ml. of 0·1N hydrochloric acid, and dilute to 1 l. with water.

3. *Copper sulphate, 5% solution.*

4. *Sodium tungstate, 6% solution.*

5. Solutions as for blood sugar estimation by Benedict's method (p. 332).

METHOD. 5 ml. of starch paste and 2 ml. of acid sodium chloride solution are transferred to each of two tubes, which are placed in a water-bath at 40° C. To one tube (*a*) is added 1 ml. of serum and to the other (*b*) 1 ml. of water. To a third tube (*c*) is added 7 ml. of water and 1 ml. of serum. The three tubes are then incubated for exactly 30 minutes. To each is then added 1 ml. of copper sulphate solution and 1 ml. of sodium tungstate solution, and after mixing the resulting precipitates are removed by filtration. 2 ml. samples of each of the filtrates are then estimated for reducing power by the blood-sugar method (see p. 332) and the result is expressed in terms of milligrams of glucose per 100 ml. of serum. Diastase activity is then expressed as: tube (*a*)—[tubes (*b*)+(*c*)].

2. *Phosphatases*

(a) *Using Glycerophosphate.* [*]

(i) *Alkaline Phosphatase.*

PRINCIPLES. A substrate of sodium glycerophosphate at pH 6·0 is acted upon by serum, and the inorganic phosphate set free is subsequently determined by the molybdenum blue method given on p. 314.

REAGENTS.

1. *Sodium glycerophosphate.* In a 100 ml. volumetric flask place 3 ml. of petroleum ether, b.p. 30° C., 80 ml. of water, 0·5 g. of sodium glycerophosphate, 0·424 g. of sodium diethyl barbiturate, and dilute with water to 100 ml. After mixing adjust to pH 8·6 with N hydrochloric acid or caustic soda.

2. *Trichloracetic acid, 10% solution.*

3. Reagents as for estimation of phosphorus in blood (see p. 314).

METHOD (*as applied to blood*). 1 ml. of serum (which should have been kept in ice to prevent enzyme action) is transferred to a test-tube, to which

* Bodansky, A., *Am. J. Clin. Path. Tech.*, Supp., 1937, **1**, 51.

are added 4 ml. of 10% trichloracetic acid. After shaking and standing for about 15 minutes, the precipitate is filtered off. This filtrate is used for the subsequent determination of inorganic phosphate.

10 ml. of the sodium glycerophosphate substrate are transferred to a glass-stoppered test-tube and placed in a water-bath at 37° C. until equilibrium is reached. 1 ml. of serum is then added to the tube, and after mixing the contents by careful inversion, the tube is placed in a 37° C. incubator for exactly 1 hour. The tube is then cooled in iced water for 2 minutes, and 9 ml. of trichloracetic acid are added to precipitate proteins. 4 ml. of the resulting filtrate are then estimated for phosphate content according to the method given on p. 314, and the result is expressed in milligrams of phosphate per 100 ml. of serum. The difference between this figure and that of the inorganic phosphate of the serum which has not been incubated gives the units of alkaline phosphatase activity.

(ii) *Acid Phosphatase.*

PRINCIPLES. The result given under "alkaline phosphatase" is due to phosphomomoesterases with optima on the alkaline side of neutrality. The acid phosphatases which are also present in blood serum have their optima at pH less than 7·0. Their concentration is determined by subjecting the serum to a glycerophosphate substrate at pH 6·4 with subsequent determination of the inorganic phosphate set free.

REAGENT.

Sodium glycerophosphate. The same proportions are used as for the alkaline phosphatase substrate except that the final mixture is adjusted to pH 6·4.

METHOD. 1 ml. of serum is added to a tube containing 10 ml. of acid substrate warmed to 37° C. Incubation of this mixture is allowed to proceed for 1 hour, at the end of which time, after cooling on ice, 9 ml. of 10% trichloracetic acid are added. 7 ml. of the filtrate are transferred to a test-tube and the phosphate content estimated as on p. 314. This result, expressed in milligrams of phosphate per 100 ml. of serum, is corrected for initial inorganic phosphate content.

(b) *Using Sodium Phenolphthalein Phosphate.**

PRINCIPLES. Phenolphthalein phosphate is hydrolysed by the enzyme, and the alkaline colour of the indicator thus produced is used for its determination.

REAGENTS.

1. *Acid substrate, pH 5·4:* 11·7 g. of sodium acetate ($3H_2O$), 0·79 ml. of glacial acetic acid, and 0·608 g. of sodium phenolphthalein phosphate are

* Huggins, C., and Talalay, P., *J. Biol. Chem.*, 1945, **159**, 399.

dissolved up to a volume of 1 l. with distilled water.   7·5 ml. of chloroform are added to preserve.

2. *Alkaline substrate*, pH 9·7 :   20·6 g. of sodium barbital and 0·608 g. of sodium phenolphthalein phosphate are dissolved up to 1 l. with distilled water and 7·5 ml. of chloroform are added.

3. *Glycine buffer*, pH 11·2 :   100 g. of carbonate-free sodium hydroxide are dissolved in 100 ml. of water.   15 ml. of this solution are mixed in 800 ml. of water with 9·19 g. of glycine, 7·17 g. of sodium chloride, and 40 g. of crystalline sodium pyrophosphate ($Na_4P_2O_7,10H_2O$).   The whole is then made up to 1 l.

4. *Phenolphthalein standard*. 100 mg. of phenolphthalein are dissolved in 100 ml. of 95% ethyl alcohol; dilute for use.

METHOD. Set up three tubes, each containing 0·5 ml. of the enzyme solution (e.g. blood plasma or serum).   To (i) add 5 ml. of water and 4·5 ml. of glycine buffer.   To (ii) add 5·0 ml. of acid substrate, and to (iii) add 5·0 ml. of alkaline substrate.   Maintain tubes (ii) and (iii) for 1 hour at 37° C., cool, and immediately add 4·5 ml. of glycine buffer.   Compare the pink colours against standards, using samples of from 10 μg. up to 50 μg. of phenolphthalein in 5 ml. of fluid plus 5 ml. of glycine buffer.

The number of milligrams of phenolphthalein liberated per 100 ml. of substrate is expressed as "units phosphatase" for each pH.

*(c) Using p-Nitrophenyl Phosphate.**

PRINCIPLES. p-Nitrophenyl phosphate (colourless) is hydrolysed to p-nitrophenol (yellow) by phosphatases.   The method is very rapid and direct, and can be carried out on 0·005 ml. of serum without any prior treatment.

REAGENTS.

1. Dissolve 7·50 g. of glycine and 0·095 g. of magnesium chloride in 700 ml. of water, add 85 ml. of 0·1N sodium hydroxide, and complete to 1 l. with water.

2. Dissolve 0·4 g. of di-sodium p-nitrophenyl phosphate in 100 ml. of N/1,000 hydrochloric acid.   Remove free phenol by extracting with butyl alcohol and then ether.

3. Mix equal parts of solutions (1) and (2) and adjust to pH 10·4. Discard when colour appears.

METHOD. To a volume of serum in a small tube (e.g. 5 cu. mm., 10 cu. mm., or 0·1 ml.) add ten times its volume of reagent (3).   Place in a water-bath at 30° C. for 30 minutes, and then immediately add a volume of N/50 sodium hydroxide equivalent to 200 times the volume of serum taken. This arrests the enzyme reaction and develops the colour of the freed phenol.

* Bessey, O., Lowry, O., and Brock, M., *J. Biol. Chem.*, 1946, **164**, 321.

Comparison of the colour is made against *p*-nitrophenol standards in alkaline solution, due allowance being made for the blank colour of reagents to which no serum has been added.

3. *Trypsin**

PRINCIPLES. Diazotised arylamines couple with proteins in alkaline solution to give chromophoric protein derivatives. These, on digestion with proteolytic enzymes, give coloured products soluble in trichloroacetic acid and suitable for colorimetric estimation.

REAGENTS.

1. *Sulphanilamide-azo-casein.*

(*a*) Dissolve 50 g. of casein (fat-free) in 1 l. of 0·1% sodium bicarbonate solution.

(*b*) Dissolve 5 g. of sulphanilamide in 200 ml. of water containing 6 ml. of 5*N* sodium hydroxide; add 2·20 g. of sodium nitrite, and stir. Add 18 ml. of 5*N* hydrochloric acid, and stir for two minutes. Then add 18 ml. of 5*N* sodium hydroxide and immediately, with stirring, add the whole of the product to solution (*a*). Then make acid to *p*H 4·5 to precipitate the azo-protein, separate, wash with water, and air dry.

2. *Substrate solution.* Dissolve 2·50 g. of azo-protein in 50 ml. of 1·0% sodium bicarbonate solution at 60° C., adjust to *p*H 8·3, and dilute to 10 ml. with water; store at 0° C.

3. *Bicarbonate buffer, *p*H 8·3.* A 0·5% solution of sodium bicarbonate.

4. *Trichloroacetic acid.* A 5% solution in water.

METHOD. Dilute 1 ml. of centrifuged duodenal juice to 100 ml. with bicarbonate buffer. Allow the substrate and diluted juice to reach 38° C. and then mix together 1 ml. of each in a small tube and maintain at 38° C. for 30 minutes. At the same time incubate similarly a tube containing 1 ml. of buffer and 1 ml. of substrate.

At the end of the reaction period add to each tube 8 ml. of 5% trichloroacetic acid to precipitate undigested protein, and filter. To 5 ml. of each filtrate are added 5 ml. of *N*/2 sodium hydroxide and the colours are measured against standards.

4. *Glycuronidase†*

PRINCIPLES. Phenolphthalein-β-glycuronidide is hydrolysed with glycuronidase and the free phenolphthalein is measured colorimetrically.

REAGENTS.

1. *Phenolphthalein-β-glycuronidide* (obtained from the urine of rabbits which have been injected with phenolphthalein); separate as the cinchonidine salt. Dissolve 0·788 g. of the cinchonidine salt in 2*N* hydrochloric

* Charney, J., and Tomarelli, R., *J. Biol. Chem.*, 1947, **171**, 507.

† Talalay, P., Fishman, W., and Huggins, C., *J. Biol. Chem.*, 1946, **166**, 757.

acid and extract with several portions of ethyl acetate. Evaporate off the solvent *in vacuo* and dissolve the residue in 100 ml. of water after neutralising free acid with sodium hydroxide.

2. *Glycine buffer, pH* 10·45. Dissolve 16·30 g. of glycine and 12·65 g. of sodium chloride in distilled water, add 10·9 ml. of sodium hydroxide solution (made by dissolving 100 g. of sodium hydroxide in 100 g. of distilled water), and complete to 1 l. with distilled water.

3. *Phenolphthalein standard.* Dissolve 100 mg. of phenolphthalein in 100 ml. of 95% ethyl alcohol.

4. *Acetate buffer, pH* 4·5. 5·785 g. of sodium acetate ($3H_2O$) and 3·25 ml. of glacial acetic acid are dissolved and made up to 1 l. with distilled water.

METHOD. 4·0 ml. of acetate buffer are pipetted into each of two test-tubes. To one tube is added 0·5 ml. of phenolphthalein-β-glycuronidide solution, and, after the tubes have reached 38° C., 0·5 ml. of enzyme solution is added to each. The tubes are stoppered and incubated for 10 minutes. At the end of this time 5 ml. of glycine buffer are added to each tube and 0·5 ml. of substrate is added to the control tube. Precipitated proteins are removed by centrifuging and the colorimetric estimation is then made.

5. *Esterase**

PRINCIPLES. The colourless acyl esters of *p*-nitrophenol are hydrolysed by esterases to produce components coloured in alkaline solution. Extremely minute amounts of esterases can be measured.

REAGENTS.

1. *Phosphate buffer, pH* 7·0, $M/15$.

2. *Sodium hydroxide, a* $N/10$ *solution.*

3. *p-Nitrophenol standards.* Aqueous solutions containing from 10 to 50 micro-moles per litre.

4. *Substrate.* 65 mg. of *p*-nitrophenyl propionate are dissolved in 10 ml. of pure methanol. 1 ml. of this solution is diluted to 100 ml. with water before use. Alternative substrates are the corresponding acetate, *iso*-butyrate or *iso*-valerate esters.

METHOD. Immediately before estimation, a solution of substrate containing exactly 0·333 micro-mole per millilitre is prepared. The concentration of substrate is arrived at by pipetting 1 ml. into 9 ml. of phosphate buffer and measuring the depth of colour of the blank. 1 ml. is also pipetted into 9 ml. of $N/10$ sodium hydroxide, in which immediate hydrolysis occurs, and the colour is again measured. The difference in tint represents colour due to hydrolysed ester.

* Huggins, C., and Laprides, J., *J. Biol. Chem.*, 1947, **170**, 425.

To perform the estimation, 1 ml. of the unknown solution is placed in a test-tube, 2 ml. of phosphate buffer and 5 ml. of water are added, and the contents are allowed to equilibrate at 25° C. 2·0 ml. of substrate are then added and a colorimetric reading is made immediately. The tube is then maintained at 25° C. for exactly 20 minutes and then a second colorimetric reading is made immediately. The difference in readings is a measure of esterase activity.

6. *Phenol Sulphatase**

PRINCIPLES. Phenols are detoxicated in organisms partly by conjugation to glycuronidide and partly to the sulphate ester.

The sulphate-splitting enzyme may be estimated by allowing it to react with *p*-nitrophenyl sulphate and measuring the *p*-nitrophenol which is liberated.

7. *Peroxidase*†

PRINCIPLES. A reaction system is used which consists of hydrogen peroxide, ascorbic acid, and *o*-tolidine. Under the action of the peroxidase, oxygen liberated from hydrogen peroxide is carried, *via* ascorbic acid, to *o*-tolidine with the production of a yellow colour.

8. *Phenol Oxidase*‡

PRINCIPLES. When mixtures of α-naphthol and dimethyl-*p*-phenylene-diamine hydrochloride are oxidised in alkaline solution a blue indophenol colour is produced. Atmospheric oxygen causes this reaction to proceed slowly; oxidase accelerates it. Comparison of the colour produced with and without oxidase can be used for the estimation of the latter.

* Huggins, C., and Smith, D., *J. Biol. Chem.*, 1947, **170**, 391.
† Dorx, H., *Ned. Akad. Weten-Amstad.*, 1942, **45**, 715.
‡ Dye, J., *Proc. Soc. Exper. Med.*, 1927, **24**, 640.

D. GASES

MICRO-COLORIMETRIC methods are particularly useful in the estimation of traces of toxic vapours in the atmosphere. The most satisfactory procedure in vapour analysis is to aspirate the air through a solution containing a solvent or reactant for the particular gas to be estimated, and then to apply a colour reaction to the solution. The concentration of the gas may then be computed in terms of the volume of air which has been sampled.

The precautions to be taken and the apparatus best used are both dependent upon the particular gas, and information on this subject may be obtained from the very full review in Jacob's book, "The Analytical Chemistry of Industrial Poisons, Hazards and Solvents."*

For estimating some gases in very low concentrations it is often more convenient to aspirate the air through a filter-paper which has been impregnated with a suitable reagent and to measure against standards the stain produced on the paper. Both these procedures have been utilised in dealing with toxic gases encountered in industrial practice.

A series of pamphlets detailing methods elaborated by the Department for Scientific and Industrial Research, published by H.M. Stationery Office, London, describe a special hand-pump and absorption-tube apparatus which are very convenient for sampling purposes. When it is necessary to use impregnated papers, a special adaptor can be fitted to the pump for this purpose.

The following table summarises the procedures involved.

TABLE IV

D.S.I.R. METHODS FOR THE RAPID ESTIMATION OF TOXIC GASES

Gas	Concentration Measurable	Procedure
Aniline.	1 in 200,000.	10 ml. of hydrochloric acid are placed in the absorption-tube and a number of aspirations are made with the pump. The solution is treated with 2 drops of 5% bleaching-powder solution and then is boiled. 5 ml. of 5% phenol in 5% ammonia are added, and, after standing for 5 minutes, the blue colour is matched against standards. The test should be repeated, varying the number of pump strokes until a measurable colour is obtained.

* Interscience Publishers, New York, 1942.

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D.S.I.R. Methods for the Rapid Estimation of Toxic Gases—*Continued*

Gas	Concentration Measurable	Procedure
Arsine.	1 in 250,000.	Papers are prepared by immersion in 5% *mercuric chloride*. A brown stain is produced with arsine.
Benzene.	1 in 10,000.	0·5 *ml. of* 40% *formaldehyde in* 10 *ml. of concentrated sulphuric acid* is placed in the absorption-tube. An orange-brown colour is produced with benzene.
Carbon bisulphide.	1 in 120,000.	10 *ml. of absolute alcohol*, 2 *ml. of* 2% *diethylamine in benzene, and* 2 *ml. of* 0·1% *copper acetate in absolute alcohol* are placed in the absorption-tube, and the brown colour due to copper di-ethyl-di-thiocarbamate is matched against standards.
Carbon monoxide.	1 in 12,000.	Test-papers are made by impregnating filter-discs with 0·25% *palladium chloride in* 50% *acetone* and allowing them to dry. Carbon monoxide reduces to the palladous condition and produces a grey stain which is compared against standards.
Chlorine.	1 in 10^6.	10 ml. of 1% *o-tolidine in* 10% *hydrochloric acid* are placed in the absorption-tube, and the number of pump strokes required to produce a given colour tint is noted (compare p. 309).
Hydrogen cyanide.	1 in 100,000.	25 *ml. of* 1% *benzidine acetate and* 2 *ml. of* 3% *copper acetate* are mixed prior to use, and test-papers are prepared from this solution. The blue colour produced on aspiration is proportional to the cyanide content.
Hydrogen sulphide.	1 in 40,000.	Papers are prepared by dipping Whatman discs in a 10% *solution of lead acetate in* 5% *acetic acid*. The brown stain of lead sulphide is compared against standards.
Nitrogen oxides.	1 in 100,000.	5 *ml. of* 0·35% *sulphanilic acid in* 14% *acetic acid and* 5 *ml. of* 0·07% *alpha-naphthylamine in* 40% *acetic acid* are mixed prior to use. The red colour produced by aspiration is proportional to the nitrous acid concentration.
Phosgene.	1 in 10^6.	Filter-papers are impregnated with a solution containing 5% *p-dimethylaminobenzaldehyde and* 5% *diphenylamine* in absolute alcohol. The pump is fitted with a pre-treatment tube of pumice granules impregnated with sodium thiosulphate and sodium iodide in order to remove hydrochloric acid and chlorine, which interfere with the phosgene reaction. The number of pump strokes required before a given yellow stain is produced on the paper, gives the concentration of phosgene in the air.
Sulphur dioxide.	1 in 250,000.	Papers are prepared from a *starch iodide-iodate mixture* (1% starch, 1% potassium iodate, and 2% potassium iodide in 30% glycerol).

Table IV (p. 380) lists methods by means of which traces of both organic and inorganic vapours may be estimated quantitatively subsequent to absorption.

TABLE IV

COLORIMETRIC METHODS FOR QUANTITATIVE VAPOUR ANALYSIS

Substance	Absorbent	Principles of Estimation	Sensitivity	Reference
Acetone and methylene ketones.	Alcohol.	Treatment with m-dinitrobenzene and alkali to produce a red colour.	1 part per million.	Compare Yant, Pearce, and Shrenk, U.S. Bureau of Mines Reports, 1935, 3323 (compare p. 342).
Acetylene.	Ammoniacal cuprous chloride.	Acetylene forms with the absorbent a colloidal suspension, cuprous acetylide.	1 mg.	Ilosvay, Ber., 1899, 32, 2,698.
Acrolein.	Water.	Oxidation with hydrogen peroxide and treatment with phloroglucinol and hydrochloric acid to produce a red colour.	5 parts per million.	Compare Pritzker, Helv. Chim. Acta., 1928, 11, 445.
Amyl alcohol.	Water.	Reaction with sulphuric acid and vanillin to produce a colour.	10 parts per million.	Penniman, Smith, and Lawshe, Ind. Eng. Chem. (Anal. Edn.), 1937, 9, 91.
Aniline.	Dilute sulphuric acid.	Production of a colour with hypochlorite.	0·5 part per million.	Elvove, Ind. Eng. Chem., 1917, 9, 953 (see p. 347).
Arsine.	Iodine plus sodium bicarbonate.	Oxidation to arsenate and measurement of molybdenum blue colour.	5 μg.	Milton, R., and Duffield, W., Analyst, 1942, 67, 279 (compare p. 278).
Benzene (also Toluene and Xylene).	Nitric and sulphuric acids.	Benzene is converted to the m-dinitro derivative. This produces with butanone and alkali a red colour.	5 μg.	Milton, R., Brit. J. Ind. Med., 1945, 2, 36 (see p. 342).
Bromine.	Potassium hydroxide solution.	Hypobromite is oxidised with hydrogen peroxide and the bromide is measured colorimetrically as bromo-sulphon-phthalein.	10 μg.	Compare p. 308.
Carbon bisulphide.	Potassium hydroxide in absolute alcohol.	Formation of potassium ethyl xanthate and colorimetric estimation of the copper salt.	10 μg.	Moskowitz, Siegl, and Burke, N.Y. State Ind. Bull., 1940, 19, 33.
Carbon dioxide.	Solution of sodium salt of phenol-phthalein.	The absorbent decreases colour intensity with increasing carbon dioxide concentration.	1 mg.	Emment, J. Ass. Off. Agr. Chem., 1931, 14, 386.
Carbon monoxide.	Palladious chloride solution.	Carbon monoxide forms metallic palladium with palladious chloride, the excess of which is measured colorimetrically after addition of potassium iodide.	0·5 mg.	Christman, Bloch, and Schultz, Ind. Eng. Chem. (Anal. Edn.), 1937, 9, 153.

COLORIMETRIC METHODS FOR QUANTITATIVE VAPOUR ANALYSIS—*Continued*

Substance	Absorbent	Principles of Estimation	Sensitivity	Reference
Carbonyls [e.g. $Ni(CO)_4$].	A heated silica tube.	Carbonyls are decomposed by heating and the metal may be dissolved and estimated by an appropriate technique.	1 μg.	Jacobs, "Analytical Chemistry of Industrial Poisons, Hazards and Solvents" (Interscience Publishers, N.Y., 1944), p. 239.
Chlorine.	o-Tolidine solution.	Chlorine produces a yellow colour with o-tolidine.	10 μg.	Porter, *Ind. Eng. Chem.*, 1926, **18**, 730 (see p. 309).
Chloroform and trichlorethylene.	1% tartaric acid in 70% alcohol.	Treatment with caustic soda and pyridine produces a pink colour on heating.	0·1 mg.	After Ross, *J. Biol. Chem.*, 1923, **58**, 641 (see p. 341).
Fluorine.	Potassium hydroxide in paraffin-coated container.	Bleaching effect of fluorides on zirconium alizarin lake measured colorimetrically.	5 μg.	De Boer, J. H., *Chem. Weekblad*, 1924, **21**, 204.
Formaldehyde.	Phenyl-hydrazine hydrochloride.	Action of formaldehyde phenyl-hydrazone with potassium ferricyanide to produce a magenta colour.	1 part per million.	Kersey, Maddocks, and Johnson, *Analyst*, 1940, **65**, 203.
Glycols.	Water.	Oxidation with periodic acid to formaldehyde. Estimation with Schiff's reagent.	5 parts per million.	Feigl, "Qualitative Analysis by Spot Tests" (N.Y., 1939) (compare p. 318).
Isopropyl alcohol.	Water.	Treatment with mercuric sulphate produces a yellow precipitate which may be estimated nephelometrically.		Jacobs, "Analytical Chemistry of Industrial Poisons, Hazards and Solvents" (Interscience Publishers, N.Y., 1944), p. 487.
Methyl alcohol.	Water.	Oxidation with permanganate and phosphoric acid to formaldehyde and colorimetric estimation with Schiff's reagent.	5 parts per million.	Jephcott, *Analyst*, 1935, **55**, 588 (compare p. 318).
Naphthalene.	Dilute picric acid solution.	Naphthalene forms an insoluble picrate which may be measured nephelometrically if a protective colloid be used.	1 mg.	Modify from Coleman and Smith, *J. Soc. Chem. Ind.*, 1900, **19**, 128.
Nitro-glycerine.	Alcohol.	Hydrolysis and estimation of nitrate with m-xylenol.	1 part per million.	Yagoda, *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15**, 27.

COLORIMETRIC METHODS FOR QUANTITATIVE VAPOUR ANALYSIS—*Continued*

Substance	Absorbent	Principles of Estimation	Sensitivity	Reference
Phenols.	Water.	Treatment with 2 : 6 di-bromoquinone chloro-imide to form indophenol.	0·05 part per million.	Gibbs, *J. Biol. Chem.*, 1927, **72**, 649 (see p. 340).
Phosgene.	Nitroso-diethylamino-phenol in xylene.	With the absorbent phos-gene produces a green colour.	0·5 µg.	Cox, H. E., *Analyst*, 1939, **64**, 807.
Phosphine.	Mercuric chloride solution.	Formation of brown col-loidal $P(HgCl)_3$.	10 µg.	Wilmet, *Analyst*, 1927, **52**, 558.
Selenium.	48% hydro-bromic acid containing free bromine.	Selenium is precipitated in colloidal form from the oxidising solution with hydroxylamine in the presence of a protective colloid.	10 µg.	Dudley, *Am. J. of Hygiene*, 1936, **24**, 227.
Sulphur chlorides.	Sodium hydroxide solution.	Oxidation to sulphate and measurement (as under sulphur trioxide below).	50 µg.	Kahn and Lei-boff, *J. Biol. Chem.*, 1928, **80**, 623.
Sulphur dioxide.	Hydrogen peroxide.	Sulphite is oxidised to sulphate and estimated by the benzidine method above.	50 µg.	Thomas, *Ind. Eng. Chem.* (Anal. Edn.), 1932, **4**, 253 (see p. 313, and as SO_3 above).
Sulphur trioxide.	10% sodium hydroxide solution.	Precipitation with benzi-dine and diazotisation of the benzidine.	50 µg.	Kahn and Lei-boff, *J. Biol. Chem.*, 1928, **80**, 623 (com-pare p. 313).
Sulphuretted hydrogen.	Zinc acetate and sodium hydroxide.	Action with ferric chlo-ride and *p*-aminodi-methylaniline to form methylene blue.	50 µg.	Sheppard and Hudson, *Ind. Eng. Chem.* (Anal. Edn.), 1930, **2**, 73 (see p. 311).
Tri-*o*-cresyl phosphate.	Water.	Hydrolysis to phenol and estimation by the diazo reaction.	5 parts per million.	Jacobs, "Analy-tical Chemistry of Industrial Poisons, Hazards and Solvents" (Inter-science Publishers, N.Y., 1944), p. 521.
Turpentine.	95% alcohol.	Turpentine produces with vanillin in concentrated hydrochloric acid a pink colour which changes to blue-green.	1 mg.	Bogatski and Biber, *J. Chem. Ind. Moscow*, 1928, **5**, 645.

E. GROUP ANALYSES

COLORIMETRIC methods are particularly suitable for the analysis of a group of substances occurring together in one sample.

Very often the sample may be oxidised or opened up as a preliminary process, and the specific analyses can then be conducted on aliquot amounts of the solution. As pointed out on p. 224, colorimetric methods applied to group analyses, especially if subsequently measured on a photo-electric instrument, may be carried out by using the minimum of apparatus and bench space, and often result in considerable time saving. Thus it is possible to introduce mass-production technique into the analytical laboratory. This is beautifully exemplified in the monograph by Vaughan* on the use of the Spekker absorptiometer in steel analysis.

The applicability of colorimetric methods to group analysis is specially valuable when a particular reagent may be used in the separation and estimation of each member of a group of substances. Thus dithizone can be used to separate and estimate several metals of the sulphide group.

1. Colorimetric Methods in Metallurgic Analysis*

(a) Composite Method for the Estimation of Molybdenum, Chromium, Manganese, and Vanadium in Steels (titanium absent)

2·0 g. of sample (steel filings) are heated with 80 ml. of "Spekker acid"† to decompose carbides, and then oxidised with sufficient nitric acid until solution is complete. It is finally fumed to remove nitric acid, cooled, and diluted to 200 ml. with water. Aliquots are then treated as follows:

A Molybdenum	B Molybdenum blank	C Manganese	D Chromium, Vanadium
Take 10 ml. To this add 10 ml. of 40% H_2SO_4, 10 ml. of 5% NaCNS, and 10 ml. of 7·5% $SnCl_2$ in 2% KI.	Conduct exactly as A, but replace 10 ml. of 5% NaCNS by 10 ml. of distilled water.	Take 50 ml. Oxidise with $AgNO_3$ (50 ml. of 0·4%) and persulphate (2·5 g.). After boiling for 3 min. cool, add 0·5 g. of urea to destroy silver complex, and complete to 100 ml. with water.	Take 100 ml. Dilute to 200 ml. and read on a violet screen (*c*). Then $b^1 - c -$ *colour due to chromium.*
After standing for 15 min. read in Spekker, using a green filter.		*(a)* Colour due to Mn (as permanganate) is read (green filters).	
$A - B =$ *colour due to Molybdenum.* Compare on calibrated graph prepared from standard solutions.		*(b)* Now reduce by adding 0·1 ml. of 5% $NaNO_2$ and again read colour. This reading is the Mn blank. Residual colour is due to Cr (as dichromate) and is read using a violet screen (b^1).	
		$a - b =$ *colour due to Manganese.*	

The D column sub-table:

Vanadium	Vanadium blank.
Take 50 ml. Add 2 ml. of H_2O_2 and read using a violet screen (*d*).	Take 50 ml. Add 2 ml. of water and read (*e*).
$d - e =$ *colour due to Vanadium.*	

* "The Use of the Spekker Absorptiometer in Metallurgical Analysis," E. J. Vaughan (Pub. by Royal Institute of Chemistry, London, 1941).

† "Spekker acid" is made as follows: 150 ml. H_2SO_4, sp. gr. 1·84; 150 ml. H_3PO_4, sp. gr. 1·75; water to 1 l.

(b) Estimation of Phosphorus in Steel

Phosphorus in steel must be determined after a different acid treatment, since "Spekker acid" contains phosphoric acid.

Treat 0·5 g. of sample with 10 ml. of sulphuric acid, 5 ml. of nitric acid, and 40 ml. of distilled water. After solution and removal of nitric fumes, add 5 ml. of 1% potassium permanganate and reduce with sulphur dioxide. Then evaporate to fuming, cool, treat with 1 ml. of hydrobromic acid, and then agitate.

Remove hydrobromic acid by heating to fuming, and, after cooling, dilute to 80 ml. with water. Boil to dissolve sulphates and then complete to 100 ml.

To 20 ml. of this solution add 60 ml. of water, 10 ml. of 5% ammonium molybdate solution and 10 ml. of 1·5% stannous chloride in 2·5% hydrochloric acid. After mixing and standing for 15 minutes, read, using a red filter (A).

Treat a further 20 ml. of solution with 70 ml. of water and 10 ml. of stannous chloride, and allow to stand for the same time period before reading (B).

Reading (A—B)=colour due to phosphorus.

(c) Estimation of Iron, Manganese, and Titanium in Aluminium Alloys*

1·0 g. of sample is digested with 40 ml. of 10% sodium hydroxide and poured into 20 ml. of water. 20 ml. of concentrated nitric acid are added, and the mixture is simmered to dissolve all solids and to remove nitrous fumes. After cooling it is diluted to 100 ml. with water. Aliquots are then treated as follows:

A	B	C	D	E
Iron	*Iron blank*	*Manganese*	*Titanium*	*Titanium blank*
Take 10 ml. Add 45 ml. of water, 40 ml. of 30% H_2SO_4, and 5 ml. of 20% NaCNS. Read, using blue-green filter. *A—B=colour due to Fe* and is compared on graph calibrated from standard solution.	Take 10 ml. Add as for *A*, replacing 5 ml. of NaCNS by 5 ml. of water.	Take 25 ml. Add 0·5 g. KIO_4 and boil. After cooling dilute to 100 ml. and read, using green filter. Then decolorise with 0·1 ml. of 2% sodium nitrite and again read, giving blank (C^1). *C—C^1=colour due to Mn.*	Take 20 ml. Add 4 ml. of 25% H_2SO_4 and 1 ml. of H_2O_2. Read, using violet filters. *D—E=colour due to Ti.*	Take 20 ml. Add 4 ml. of 25% H_2SO_4 and 1 ml. of water, and read.

2. Colorimetric Methods in Blood Analysis

This schema was originally due to Folin and Wu, but has been subsequently added to and modified by various workers. In essence it consists of deproteinisation with tungstic acid and analysis on the clear filtrate for various substances.

* Vaughan, E. J., loc. cit.

5 ml. of blood are run into 45 ml. of a mixture of 5 ml. of 10% sodium tungstate and 40 ml. of $N/12$ sulphuric acid. After vigorously shaking the mixture is filtered. Aliquots are then treated as follows:

A^1	B^2	C^3	D^4	E^5	F^6
Uric acid	Urea	Non-protein nitrogen	Glucose	Amino nitrogen	Creatine
Take 5 ml. of filtrate and estimate by Benedict's direct method (p. 347), using arseno-molybdic reagent (p. 333).	Take 5 ml. of filtrate and use urease to convert ammonia. Follow by aeration and Nesslerisation (p. 297).	Take 5 ml. of filtrate, decompose by Kjeldahl's method (p. 78), and treat with Nessler's reagent (p. 297).	Take 2 ml. of filtrate and estimate by the Benedict method, using copper reduction and the molybdate colour (p. 333).	Take 5 ml. of filtrate and treat with sodium β-naphthoquinone sulphonate (p. 348).	Take 5 ml. of filtrate and autoclave with 1 ml. chloric acid for 10 min. at 155° C. Then add 5 ml. of picric acid and 1 ml. of 10% sodium hydroxide.

1. Benedict, S., *J. Biol. Chem.*, 1922, **54**, 233.   2. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.
3. Koch, F., and McMeekin, J., *J. Amer. Chem. Soc.*, 1924, **46**, 2066.
4. Benedict, S., *J. Biol. Chem.*, 1931, **92**, 141.
5. Folin, O., *J. Biol. Chem.*, 1922, **51**, 377.   6. See 2.

3. Estimation of the Normal Minerals in Foodstuffs*

Foodstuffs are oxidised with nitric acid/ammonium nitrate mixture, and the dissolved product is estimated for inorganic constituents using aliquot portions of the solution.

A 1·0 g. sample is oxidised with nitric acid/ammonium nitrate mixture (p. 101), evaporated to fuming, diluted, boiled, and taken up in 10 ml. of dilute hydrochloric acid. Aliquots are then treated as follows:

A	B	C	D	E	F	G
Calcium	Sulphate	Sodium	Potassium	Phosphorus	Iron	Magnesium
Precipitate as oxalate, dissolve in H_2SO_4, add excess of $KMnO_4$ and estimate colorimetrically (p. 292).	Precipitate with benzidine and estimate colorimetrically (p. 313).	Precipitate with magnesium uranyl acetate and estimate colorimetrically (p. 296).	Precipitate with sodium cobaltinitrite and estimate the cobalt colorimetrically with alpha-nitroso-β-naphthol (p. 291).	Estimate directly as molybdenum blue (p. 314).	Estimate with o-phenanthroline (p. 287).	Precipitate as $MgNH_4PO_4$ and estimate the precipitated phosphate colorimetrically (p. 314).

4. Estimation of Heavy Metal Contaminants of Foods or Drugs†

Arsenic, Copper, Lead, Zinc, Iron, Bismuth, Nickel, and Cadmium

The method is based upon (i) the use of diethyl-dithiocarbamate (see p. 48) to remove, by selective extraction with chloroform, copper, bismuth, mercury, and the noble metals, followed by (ii) separation of lead, zinc,

* Milton, R., Hoskins, J., and Jackman, W., *Analyst*, 1944, **69**, 99.
† Strafford, N., Wyatt, P., and Kershaw, F., *Analyst*, 1945, **70**, 232.

cadmium, nickel, and iron using the dithizone reagent (p. 45) in toluene. By using a weak dithizone solution under controlled conditions very sharp separation may be obtained. Tervalent arsenic is extractable with the carbamate reagent, quinquevalent arsenic is not; this allows of separation of arsenic from copper.

By the procedure given below all interference from common metals in concentrations of up to 500 parts per million is eliminated.

REAGENTS.

1. *Ammonia solution*, $10N$ (free from metals).

2. *Diethyl dithiocarbamate.* (i) Stock solution—dilute 3 ml. of redistilled diethylamine to 10 ml. with chloroform (B.P.) and add slowly, with stirring, 1 ml. of redistilled carbon disulphide previously diluted to 10 ml. with chloroform. (ii) Extraction reagent—dilute 5 ml. of stock solution to 100 ml. with chloroform.

3. *Dithizone solution.* (i) 0·001% solution in toluene—dissolve about 15 mg. of dithizone in 50 ml. of redistilled toluene. Shake in a 100 ml. separating funnel with 50 ml. of water containing 2 ml. of the ammonia solution. Reject the toluene layer. Acidify the aqueous layer slightly with $5N$ hydrochloric acid and extract with 50 ml. portions of redistilled toluene. Combine the toluene extracts and wash with 10 ml. portions of water. Prepare freshly as required. (ii) 0·008% solution in chloroform— prepare as above, substituting chloroform for toluene.

4. *Sodium citrate* (approximately $1M$ solution). Dissolve about 150 g. of trisodium citrate (A.R.) in water, add 0·5 ml. of concentrated ammonia, dilute to 500 ml., and shake with 25 ml. portions of 0·02% dithizone in chloroform until the last extract remains green and the aqueous solution is slightly yellow. Add 5 ml. of 20% citric acid (A.R.) and extract with 25 ml. portions of dithizone until colourless.

5. *Potassium cyanide* (approximately $1·5M$). Dissolve 50 g. of potassium cyanide (A.R.) in the minimum amount of water, dilute to 100 ml., and extract with 10 ml. portions of dithizone (0·02% in chloroform) until the last portion remains green and the aqueous layer is slightly yellow. Extract the excess dithizone with chloroform and dilute the extracted solution to 500 ml. with water. This reagent can be used for a period of about 6 months before renewal is necessary.

6. *Potassium iodide*, 20% *solution.* Dissolve 20 g. of potassium iodide (A.R.) in 100 ml. of water, add 0·2 ml. of ammonium hydroxide solution, extract with 10 ml. of carbamate reagent and shake for 30 seconds. Reject the chloroform layer and wash with two 5 ml. portions of chloroform.

7. *Acid molybdate solution.* Mix exactly 250 ml. of standardised 11·0N sulphuric acid with 250 ml. of 7% ammonium molybdate solution. Filter, wash with water, add exactly 250 ml. of standardised 3·0N perchloric acid,

and dilute to 1 l. with water at 20° C. Mix well. 5 ml. of this solution should require 17·5 ml. N sodium hydroxide when titrated to methyl red indicator.

8. *Sodium metabisulphite, 5% in water, filtered.*

9. *Stannous chloride, 0·4%.* Dilute to 50 ml. with water 1·0 ml. of a 20% w/v solution of stannous chloride in concentrated hydrochloric acid. Prepare the dilute solution freshly as required and make a fresh stock solution each week.

10. *Formaldehyde, 40% w/v, filtered.*

METHOD

(a) *Wet decomposition.*

Decompose 2 g. of the sample in a modified Kjeldahl flask with sulphuric, nitric, and perchloric acids (p. 101).

(b) *Extraction of Cu, Bi, Hg* (noble metals).

Dilute the solution and add hydrochloric acid until its concentration is not less than $2N$. Cu, Bi, Hg, etc., may then be extracted in the cold with carbamate reagent. Some Fe is also removed, but As^V is not extracted.

1st Carbamate extract (A) Decompose with sulphuric and perchloric acids (p. 101), add iodide and metabisulphite, re-extract Bi, Cu, etc., with carbamate reagent. [Extract (E).]	*Acid layer (B)* Reduce to As^{III} with iodide and metabisulphite and extract with carbamate reagent.

Acid layer (AI)
This contains residual Fe. Add it to acid solution BII (see across).

Carbamate extract (E)
Decompose with sulphuric and perchloric acids and dilute. (AII)
Bismuth—treat (AII) with citrate and metabisulphite, neutralise, add potassium cyanide to prevent extraction of Cu, etc., and extract Bi with dithizone in toluene ; wash with potassium cyanide and determine the optical density.
Copper—after extracting Bi with dithizone, add formaldehyde to the aqueous layer and extract Cu with carbamate reagent. Clarify with anhydrous sodium sulphate and read the optical density.

2nd Carbamate extract (BI) *Arsenic*—evaporate chloroform and add molybdate reagent. Oxidise, reduce with metabisulphite, and dilute to 5 ml. with water. Add 0·5 ml. of stannous chloride and measure the blue colour.	*Acid layer (BII)* Evaporate (BII) and (AI) together with citrate and metabisulphite. Dilute, treat with citrate and metabisulphite, neutralise with ammonia, and extract Pb, Zn, Hg, Cd, and Co with dithizone in toluene.

Dithizone extract Extract Pb, Zn, Cd with $N/10$ acid citrate solution.	*Aqueous layer* Determine iron colour with thioglycollate or o-phenanthroline (p. 287).

Dithizone layer (BIII) Ni(Co) *Nickel*—decompose solution with sulphuric, nitric, and perchloric acids. Add citrate, bromine water, and ammonia. Then add dimethyl glyoxime. Determine the optical density at 25 ml. dilution in a 4 cm. cell, using Spekker blue filter 7.	*Acid extract (BIV)* (Pb, Zn, Cd) *Lead*—treat extract with citrate, ammonia, and potassium cyanide. Extract and determine Pb as dithizonate. *Extraction of Zn, Cd* Treat the residue with formaldehyde. Extract Zn and Cd with dithizone in chloroform. *Determination of Zn and Cd* Wash extract with dilute ammonia, determine optical density of mixed dithizonates at 20 ml. dilution in a 1 cm. cell, using Ilford green filter No. 604. Treat 10 ml. portion with sodium hydroxide to decompose Zn dithizonate, and measure the optical density of the residual Cd dithizonate (filter 604). Zn is obtained by difference.

5. Colorimetric Methods for Water Analysis

Colorimetric methods are particularly useful for routine analyses of potable water, since, with the relatively simple nature of the medium, interference is at a minimum.   Also, these methods are most suitable for serial analyses, and where large numbers of samples are to be analysed.

Substance	Principle	Reference
1. Free ammonia.	Water is made alkaline with sodium carbonate and distilled. The distillate is treated with Nessler's solution and the yellow colour is measured.	D i c k i n s o n, D., "Chemical Analysis of Waters" (Blackie, London, 1944), p. 38.
2. Albuminoid ammonia.	The residue from (1) is treated with alkaline permanganate, and after dilution is again distilled. The distillate is then treated with Nessler's reagent. Sensitivity: 0·2 μg.	D i c k i n s o n, D., "Chemical Analysis of Waters" (Blackie, London, 1944), p. 38.
3. Chlorine and chloramine.	o-Tolidine solution imparts a colour to water containing free chlorine or chloramine. The interfering effect of Mn, Fe, etc., may be allowed for by making a second estimation after the chlorine compounds have been destroyed with arsenite. Sensitivity: 5 μg. per 100 ml.	"Standard Methods for the Examination of Water and Sewage" (New York, 1946), p. 100.
4. Cyanide.	Conversion to thiocyanate with ammonium sulphide and measurement of the colour produced with ferric salts. The method of Aldridge (p. 309) is much more sensitive and can be applied directly to water analysis. Sensitivity: 0·3 μg. per 100 ml.	Fasker, J. E., *J. Amer. Waterworks Assn.*, 1940 **32**, 187.
5. Fluoride.	The coloured lake formed with zirconium nitrate and sodium alizarin sulphonate is bleached on the addition of fluorides. The diminution in colour may then be measured (compare p. 307). Sensitivity: 1·0 μg. per 100 ml.	Walker, O., and Caine, G., *Canadian J. of Res.*, 1945, **23B**, 275.
6. Nitrate.	The yellow colour produced when brucine in sulphuric acid is added to water may be used. See also pp. 310, 311. Sensitivity: 5·0 μg. per 100 ml.	Noll, C., *Ind. Eng. Chem.* (Anal. Edn.), 1945, **17**, 456.
7. Total nitrite and nitrate.	HNO_2 and HNO_3 are reduced to ammonia with Devarda's alloy. The Nessler technique is then followed. See also p. 311. Sensitivity: 0·5 μg per 100 ml.	Allerton, J. W., *Analyst*, 1947, **72**, 349.
8. Phosphate.	Treatment with ammonium molybdate and reduction with hydroquinone produces a blue colour. See also pp. 314–315. Sensitivity: 5·0 μg. per 100 ml.	Briggs, A., *J. Biol. Chem.*, 1924, **59**, 255.

COLORIMETRIC METHODS FOR WATER ANALYSIS—*Continued*

Substance	Principle	Reference
9. Silicate.	Addition of ammonium molybdate and reduction with sulphite at pH 2·4 allows of the estimation of silicate in the presence of phosphate. See also p. 313. Sensitivity: 10 μg. per 100 ml.	Straub, F. G., and Grabowski, H., *Ind. Eng. Chem.* (Anal. Edn.), 1944, **16**, 574.
10. Sulphide.	250 ml. of water are acidified and distilled. The distillate is treated with dimethyl-*p*-phenylenediamine and ferric chloride to produce methylene blue. See p. 311. Sensitivity: 10 μg. per 100 ml.	Dickinson, D., *Analyst*, 1945, **70**, 5.
11. Sulphate.	Sulphate is precipitated with benzidine in acetone solution. The precipitate is diazotised and coupled with thymol to produce a colour. See p. 313. Sensitivity: 2·0 μg. per 100 ml.	Cuthbertson, D., and Tompsett, S., *Biochem. J.*, 1931, **25**, 1937.
12. Phenols.	A neutralised sample of water is distilled, and the distillate is buffered to pH 9·4 and coupled with dibromoquinone-chlorimide. An indophenol blue colour is produced. Sensitivity: 2·0 μg. per 100 ml.	"Standard Methods for the Examination of Water and Sewage" (New York, 1946, Ninth Edn.), p. 217.
13. Tannins.	Tannins (and phenols) in alkaline solution reduce phospho-tungstomolybdic acid and produce a blue colour. Sensitivity: 10 μg. per 100 ml.	Berk, A., and Schroeder, W., *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 456.

NOTE.—In the majority of these analyses a greatly increased sensitivity may be obtained if a prior separation, or concentration, can be made.

ESTIMATIONS OF TRACES OF METALLIC IONS IN WATER

Substance	Principle	Reference
1. Aluminium.	Haematoxylin is added and the tint of the coloured lake is measured. Sensitivity: 5 μg. per 100 ml.	Houghton, G. U., *Analyst*, 1945, **70**, 335.
2. Arsenic.	Evolution as arsine, absorption in hypobromite and colour development with ammonium molybdate and hydrazine. Sensitivity: 5 μg. per 100 ml.	Jacobs, M., and Nagler, J., *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 442.
3. Chromium.	Chromate produces a blue colour with diphenylcarbazide. Water is evaporated with sulphuric acid to the fuming point, the residue is made alkaline and oxidised with peroxide. Excess of peroxide is removed in boiling acid solution and the colour reagent is then added. Sensitivity: 1 μg. per 100 ml.	Graham, J., *J. Amer. Waterworks Assn.*, 1943, **35**, 159.

ESTIMATIONS OF TRACES OF METALLIC IONS IN WATER—*Continued*

Substance	Principle	Reference
4. Copper.	Extract the copper with dithiocarbamate into carbon tetrachloride solution and measure the yellow colour. Sensitivity: 5 μg. per 100 ml.	Stone, I., *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 479.
5. Lead.	Water is evaporated with sulphuric and nitric acids. The lead separated as sulphate is redissolved in ammonium acetate and then measured as the brown sulphide after addition of ammonium sulphide. Sensitivity: 10 μg. per 100 ml.	Monier-Williams' "Lead in Foods,' H.M.S.O., 1938. (See p. 284).
6. Iron.	Water is treated with HCl to dissolve colloidal iron compounds, buffered with sodium acetate, reduced with hydroxylamine, and the red colour given with o-phenanthroline is measured. Sensitivity: 25 μg. per 100 ml.	Caldwell, D., and Adams, R., *J. Amer. Waterworks Assn.*, 1946, **38**, 72.
7. Magnesium.	To water are added starch solution and acid calcium sulphate as protective colloids, followed by Titan Yellow solution. With magnesium there develops, on making alkaline, an orange lake suitable for colorimetry. Sensitivity: 50 μg. per 100 ml.	Ludwig, E., and Johnson, C., *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 895.
8. Manganese.	Water is boiled with ammonium persulphate, and the permanganate colour then produced is measured. Sensitivity: 20 μg. per 100 ml.	"Standard Methods of Water Analysis" (Amer. Pub. Health Assn., New York, 1946, Ninth Edn.), p. 57.
9. Sodium.	Water is evaporated and treated with zinc uranyl acetate solution. The sodium salt is separated, dissolved in water, and the yellow colour is measured. Sensitivity: 50 μg. per 100 ml.	McCormick, D., and Carlson, W., *Chemist and Analyst*, 1942, **31**, 15. (See p. 296).
10. Selenium.	The sample is made alkaline and oxidised with sodium peroxide, evaporated in the presence of hydrogen bromide and bromine, and then distilled. The distillate is treated with hydroxylamine hydrochloride and the yellow colour is measured.	"Standard Methods of Water Analysis" (New York, 1946, Ninth Edn.), p. 210.

COLORIMETRIC ESTIMATION OF pH

Although various methods for the estimation of pH by photometric methods have been published, the visual method of comparison is probably the most satisfactory procedure. By this simple technique a pH may be measured accurately to ± 0.1 unit, and the inherent errors involved in the use of indicators (see pp. 131 to 137) make this a limit for *any* colorimetric method. If a higher accuracy is required then electrometric methods (Part V, pp. 411–414) must be used.

Briefly, the colorimetric technique consists of adding a constant amount of an appropriate indicator to a measured volume of the liquid (usually 0.05 ml., i.e. 1 drop, to every 1 ml. of solution) and also to a series of equal volumes of buffer solutions covering the pH range of the indicator. Visual matching is then made.

If the solution is initially coloured, a third tube containing the same amount of the liquid to be tested should be placed behind the standard tubes while the colour comparison is being made, so that the additive effects of the colours of the tested solution and of the indicator are compared (see pp. 236–238).

Several compact models of comparators have been designed for this purpose by manufacturers of scientific apparatus.

A more rapid and equally satisfactory method of estimating pH visually is by employing the B.D.H. "Capillator" technique.*

This consists of adding to 1 ml., or less, of the solution to be tested a standardised amount of indicator, and allowing the mixture to flow up into a clean capillary tube of given bore, which is then compared against a card containing permanent standards covering the whole pH range of any one indicator. These permanent standards are prepared accurately from buffer solutions of the pH required to cover the indicator range in intervals of 0.2 of a unit and then are sealed hermetically to prevent evaporation.

Although they are reasonably permanent, these indicator standards should be stored in the dark to minimise photo-chemical decomposition, and may in any case need renewal after a period of a few years.

The Tables on pp. 392–393 give directions for preparing standard buffers, whilst Table I of pp. 132–133 lists appropriate indicators and gives their method of preparation.

The Table on p. 393 gives details for the preparation of *Universal Indicators* for the approximate determination of pH to an accuracy of 1 unit. Permanent colour standards of tinted glass are available for use with indicator mixtures of this type, and are to be recommended for rapid routine work.

* Manufactured by British Drug Houses, Ltd., London.

TABLES OF BUFFER SOLUTIONS

The following tables give directions for preparing standard buffer solutions. Pure, recrystallised reagents and accurately standardised acid or alkali must be used.

1. *p*H *Range* 2·2–3·9.

To 50·0 ml. of 0·20*M* acid potassium phthalate (40·828 g. per litre) add the following volumes of 0·20*N* hydrochloric acid and dilute each mixture to 200 ml. with distilled water.

Vol. HCl	*p*H	*Vol.* HCl	*p*H
46·70 ml.	2·2	17·70 ml.	3·1
42·50	2·3	14·70	3·2
39·60	2·4	11·80	3·3
37·00	2·5	9·90	3·4
32·95	2·6	7·50	3·5
29·60	2·7	5·97	3·6
26·42	2·8	4·30	3·7
22·80	2·9	2·63	3·8
20·32	3·0	1·00	3·9

Suitable Indicators : Range *p*H 2·0–2·8, thymol blue.
Range *p*H 2·8–4·6, phenol blue.

2. *p*H *Range* 4·0–6·3.

To 50·0 ml. of 0·2*M* acid potassium phthalate add the following volumes of 0·20*N* sodium hydroxide and dilute each mixture to 200 ml. with distilled water.

Vol. NaOH	*p*H	*Vol.* NaOH	*p*H
0·40 ml.	4·0	23·80 ml.	5·0
2·20	4·1	27·20	5·1
3·70	4·2	29·95	5·2
5·17	4·3	32·50	5·3
7·50	4·4	35·45	5·4
9·60	4·5	37·70	5·5
12·15	4·6	39·85	5·6
14·60	4·7	43·00	5·8
17·70	4·8	45·45	6·0
20·95	4·9	47·00	6·2

Suitable Indicators : Range *p*H 4·0–4·6, bromophenol blue.
Range *p*H 4·4–6·0, methyl red.
Range *p*H 5·2–6·8, bromocresol purple.

3. *p*H *Range* 5·8–8·0.

To 50·0 ml. of 0·2*M* dipotassium hydrogen phosphate (27·231 g. per litre) add the following volumes of 0·20*N* sodium hydroxide and dilute to 200 ml. with distilled water.

Vol. NaOH	*p*H	*Vol.* NaOH	*p*H
3·72 ml.	5·8	29·63 ml.	7·0
4·70	5·9	32·50	7·1
5·70	6·0	35·00	7·2
7·40	6·1	37·40	7·3
8·60	6·2	39·50	7·4
10·19	6·3	41·20	7·5
12·60	6·4	42·80	7·6
16·00	6·5	44·20	7·7
17·80	6·6	45·20	7·8
21·00	6·7	46·00	7·9
23·65	6·8	46·80	8·0
26·50	6·9		

Suitable Indicators : Range *p*H 5·6–6·8, bromocresol purple.
Range *p*H 6·0–7·6, bromothymol blue.
Range *p*H 6·8–8·4, phenol red.

4. pH *Range* 7·8–10·0.

To 50·0 ml. of 0·2M boric acid in 0·2M potassium chloride (12.048 g. of boric acid and 14·912 g. of potassium chloride in 1 l. of water) add the following volumes of 0·20N sodium hydroxide, and dilute to 200 ml. with water.

Vol. NaOH	pH	*Vol.* NaOH	pH
2·61 ml.	7·8	21·30 ml.	9·0
3·30	7·9	24·30	9·1
3·97	8·0	26·70	9·2
4·80	8·1	29·95	9·3
5·90	8·2	32·00	9·4
7·30	8·3	34·50	9·5
8·50	8·4	36·85	9·6
10·40	8·5	39·00	9·7
12·00	8·6	40·80	9·8
14·30	8·7	42·50	9·9
16·30	8·8	43·90	10·0
19·00	8·9		

Suitable Indicators: Range pH 7·2–8·8, cresol red.

Range pH 8·0–9·6, thymol blue.

5. UNIVERSAL BUFFER MIXTURE, pH RANGE 2·0–11·94*

100 ml. of a mixture which is 0·04M in respect to each of the following acids: (i) phosphoric acid, (ii) phenylacetic acid, and (iii) boric acid are mixed with the following volumes of 0·20N sodium hydroxide, and then diluted to a total volume of 200 ml.

Millilitre NaOH	pH	*Millilitre* NaOH	pH
0·0	1·8	50·0	7·0
2·5	1·9	52·5	7·0
5·0	2·0	55·0	7·2
7·5	2·1	57·5	7·5
10·0	2·2	60·0	8·0
12·5	2·4	62·5	8·4
15·0	2·6	65·0	8·7
17·5	2·8	67·5	8·9
20·0	3·1	70·0	9·1
22·5	3·4	72·5	9·4
25·0	3·6	75·0	9·4
27·5	3·9	77·5	9·9
30·0	4·2	80·0	10·3
32·5	4·3	82·5	10·8
35·0	4·7	85·0	11·1
37·5	4·9	87·5	11·3
40·0	5·4	90·0	11·5
42·5	6·0	92·5	11·6
45·0	6·0	95·0	11·8
47·5	6·6	100·0	11·9

* Prideaux and Ward, *J. Chem. Soc.*, 1924, **125**, 426; Britton and Robinson, *J. Chem. Soc.*, 1931, 458.

TABLE OF UNIVERSAL INDICATORS
FOR APPROXIMATE pH DETERMINATION BY DIRECT INSPECTION

Composition of the Indicator	Colour Changes
Mixture A.	
0·1 g. phenolphthalein; 0·2 g. methyl red; 0·3 g. dimethylamino-azobenzene; 0·4 g. bromothymol blue; and 0·5 g. thymol blue.	*p*H 1, cherry-red; *p*H 2, rose; *p*H 3, red-orange; *p*H 4, orange-red; *p*H 5, orange; *p*H 6, yellow; *p*H 7, yellow-green; *p*H 8, green; *p*H 9, blue-green; *p*H 10, blue.
Dissolve in 500 ml. of alcohol and titrate in sufficient *N*/10 sodium hydroxide to produce a yellow colour.	
Mixture B.	
0·35 g. neutral red; 0·15 g. thymol-sulphone-phthalein; 0·25 g. thymol-phthalein; 0·10 g. nitramine; and 0·60 g. *m*-nitrophenol.	The colours follow the order of the spectrum, from red at *p*H 7 to blue at *p*H 14.
Dissolve in 1 l. of 50% alcohol.	

Mixture A is preferable for the acid range and Mixture B for the alkaline range. The indicator should be added drop by drop to the solution to be examined, and compared with standard coloured glasses in a comparator or tintometer, or against known buffered solutions in capillator tubes. If the indicator solution is not provided with its own dropping tube, a clean, dry pipette should be used each time, taking great care to avoid contamination.

REPRESENTATIVE EXAMPLES OF NEPHELOMETRIC AND FLUORIMETRIC ANALYSES

1. NEPHELOMETRIC METHODS

As explained on pp. 250–252, nephelometry depends upon measurement of the amount of light scattered by a fine suspension. All the methods described in this section may, however, be measured turbidimetrically if a photometer or photo-electric instrument is available, in which case it is presumed that the degree of light extinction is proportional to the concentration of the suspension.

(a) Nephelometric Estimation of Arsenic*

PRINCIPLES. After destruction of organic matter, arsenic is precipitated with cocaine molybdate and the dispersed turbidity is measured. Under 1 μg. of arsenic may be estimated.

Phosphate must be absent, since a cloud of similar sensitivity is produced with the same molybdate reagent. For the determination of phosphorus, a strychnine molybdate reagent is preferable, however, since this will detect 1 part of phosphorus in 300 million parts of water, and 1 part in 200 million may readily be estimated.

REAGENTS. These should all be arsenic-free as used for the estimation of arsenic by Bang's method (see p. 174).

1. *Potassium molybdate, 1% solution.*
2. *Cocaine hydrochloride, 2% solution.*
3. *Hydrochloric acid, 1N.*
4. *Perhydrol.*
5. *Nephelometric reagent.* Prior to use, mix 1 volume of potassium molybdate solution, 2 volumes of N hydrochloric acid, and add, with shaking, 1 volume of cocaine hydrochloride.

METHOD. The sample is oxidised and arsenic is distilled as the trichloride in the manner described on p. 174. The distillate is trapped in N sodium hydroxide in the receiver. The resulting sodium arsenite is treated with a few drops of perhydrol and warmed to convert it to arsenate. The distillate is then neutralised with dilute hydrochloric acid and evaporated to about 5 ml. After cooling, an equal volume of nephelometric reagent is then added, and the cloud is compared in a nephelometer with standard arsenate solutions which have been treated similarly.

* Kleinmann, H., and Pangritz, F., *Biochem. Z.*, 1927, **185**, 14.

It is essential that the standard and unknown should have closely similar concentrations, and for this purpose it is best to use a series of standards and to match against the most appropriate of them. The best results are obtained when the arsenic concentration is between 0·05 and 0·0025 mg. in the 5 ml. of solution used; with suitable precaution the method may be used for quantities as low as 0·0005 mg.

(b) Nephelometric Estimation of Chloride in Blood*

PRINCIPLES. Plasma is deproteinised by the zinc hydroxide method of Somogyi (p. 333). The filtrate is treated with a special silver nitrate/gum ghatti reagent and boiled to produce a reproducible suspension suitable for nephelometric measurement.

REAGENTS.
1. *Zinc sulphate, 0·4% solution.*
2. *N/10 sodium hydroxide.*
3. *Silver nitrate reagent.* Mix 800 ml. of 1% silver nitrate, 80 ml. of concentrated nitric acid, and 40 ml. of gum ghatti solution (p. 291). Make up to 1 l. with distilled water.

METHOD. 4·8 ml. of zinc sulphate solution and 1·0 ml. of $N/10$ sodium hydroxide are pipetted into a tube with a graduation at 6 ml. 0·2 ml. of blood or plasma is added, and, after shaking well, the tube is placed in a boiling water-bath for 3 minutes. The contents of the tube are cooled and the volume is completed to 6 ml. with water. After a further mixing the precipitated protein is removed by filtration through a dry filter-paper, and the filtrate is collected into a dry tube. 3 ml. of the clear filtrate are transferred to another tube, 3 ml. of the silver reagent are added, and the tube is heated in a boiling water-bath for 20 minutes. After cooling the tube is shaken to produce even suspension and the cloud is compared in a nephelometer against standard chloride solutions which have been treated similarly.

(c) Nephelometric Estimation of Inorganic Sulphate (in Urine)†

PRINCIPLES. If certain peptones are heated with barium chloride, a complex is formed which, on addition to a sulphate, produces a highly dispersed suspension of barium sulphate.

The effective range is from 0·1–1·0 mg. of sulphate calculated as SO_3.

REAGENTS.
1. *Peptone/barium chloride solution.* Dissolve 20 mg. of peptone‡ in 5 ml. of 1% barium chloride solution. Add a few drops of bromo-cresol purple

* Obermer, E., and Milton, R., *Biochem. Z.*, 1932, **251**, 329.
† Obermer, E., and Milton, R., *Bull. Soc. Chem. Biol.*, 1932, **14**, 1447.
‡ Armour's bacteriological beef peptone.

indicator and buffer to pH 6·0, using dilute hydrochloric acid or sodium hydroxide. Add 1 g. of sodium chloride and complete to 10 ml. with water. Heat in a boiling water-bath for 10 minutes, cool, and add a few drops of chloroform. This solution, if kept stoppered, remains stable for many months.

2. *Gum ghatti*, 2% *solution* (p. 291).

3. *Diluting mixture.* Mix 50 ml. of gum ghatti solution with 200 ml. of water and 250 ml. of 2% barium chloride solution.

4. *Nephelometric solution.* Before use mix 1 ml. of peptone/barium chloride solution with 50 ml. of diluting solution.

METHOD. 1·5 ml. of urine are measured into a test-tube. 6 ml. of 1% sodium chloride and 4·5 ml. of N hydrochloric acid are added. A knife-point of charcoal is introduced into the mixture, and after shaking vigorously for 1 minute, the tube is allowed to stand aside for 10 minutes. The contents are then twice filtered through the same paper into a dry test-tube. 4 ml. of the filtrate are pipetted into another tube, and to this are added 2 ml. of N hydrochloric acid and 4 ml. of 1% sodium chloride. After mixing, 2 ml. of the nephelometric solution are added, and the tube is set aside for 30 minutes to allow of complete reaction. At the end of this time the contents of the tube are thoroughly shaken and measured in a nephelometer against standard sulphate suspensions similarly prepared.

(d) Table of Nephelometric Methods

Exact compliance with instructional details is essential for attainment of reproducibility. The original references should therefore be consulted. More detailed information is given in Yeo's monograph.*

Estimation of	*Principle*	*Reference*
Inorganic Substances		
Ammonia.	Mercuric chloride, with sodium chloride and lithium carbonate, produces with ammonium salts a colourless cloud of $Hg(NH_2)Li$. Sensitivity: 1 part in 160 million.	Graves, S. S., *J. Amer. Chem. Soc.*, 1915, **37**, 1171.
Calcium.	With sodium sulpho-ricinoleate, calcium produces a highly dispersed soap. Sensitivity: 0·05–0·5 mg. of calcium may be measured effectively.	Rona, P., and Kleinmann, H., *Biochem. Z.*, 1923, **137**, 157.
Lead.	A precipitate suitable for nephelometric estimation is produced when potassium dichromate is added to lead salts in acetic acid solution. Sensitivity: 0·05 mg. may be estimated.	Danckwortt, P. W., and Jurgens, E., *Arch. Pharm.*, 1928, **266**, 374.

* "Photometric Chemical Analysis," Vol. II; "Nephelometry" (Chapman and Hall, London, 1929).

Estimation of	*Principle*	*Reference*
Magnesium.	Magnesium is precipitated as $MgNH_4PO_4$ and the phosphate is determined nephelometrically (see below).	Denis, W., *J. Biol. Chem.*, 1920, **41**, 362.
Phosphate.	Phosphate gives with strychnine molybdate a finely dispersed precipitate. The effective range is from 0·0005–0·1 mg. P_2O_5.	Kleinmann, H., *Biochem. Z.*, 1926, **174**, 43.
Organic Substances		
Acetone.	Acetone is precipitated by silver mercury cyanide. Sensitivity: 1 part in 100 million.	Marriott, W., *J. Biol. Chem.*, 1913, **16**, 289.
Caffeine.	Caffeine gives with concentrated sodium phosphotungstate solution a white precipitate. Sensitivity: 2 mg. per 100 ml.	Herndlhofer, E., *Mikrochemie*, 1932, **12**, 227.
Citric acid.	Citric acid when oxidised with permanganate is converted to acetone dicarboxylic acid. This latter forms an insoluble compound with mercuric sulphate. Sensitivity: 0·1 mg. may be measured.	Deniges, G., *Comp. rend. Soc. Biol.*, 1902, **54**, 197.
Nicotine.	Silico-tungstic acid produces a white turbidity with nicotine solutions. Sensitivity: 10 parts per million.	Wakeham, G., *Chem. Analyst*, 1930, **19**, 8.
Phenols.	Phenols may be estimated nephelometrically after reaction with bromine to form tribromophenols.	Shaw, J., *Ind. Eng. Chem.* (Anal. Edn.), 1929, **1**, 118.
Protein.	(i) Thiosalicylic acid produces a turbidity with proteins, though not with amino-acids, peptides, or purines. (ii) Protein fractions may be estimated by selective precipitation with ammonium sulphate.	Walker, B., and Bakst, H., *J. Lab. Clin. Med.*, 1934, **20**, 312. Rusznyak, S., *Biochem. Z.*, 1922, **133**, 370.
Purines.	Ammoniacal silver nitrate produces a white precipitate with purine bases. Sensitivity: 0·1 mg. can be detected.	Graves, S., and Kober, P., *J. Amer. Chem. Soc.*, 1915, **37**, 2430.
Urea.	Urea is precipitated with xanthydrol. The effective range is from 0·1–1·0 mg. of urea in 5 ml. of solution.	Auguste, C., *Comp. rend. Soc. Biol.*, 1925, **93**, 639.
Zinc.	Zinc is precipitated with potassium ferrocyanide.	Fairhall and Richardson, *J. Amer. Chem. Soc.*, 1930, **52**, 938.

2. FLUORIMETRIC METHODS

(a) Fluorimetric Estimation of Beryllium*

PRINCIPLES. If a solution of beryllium in alkaline solution be treated with tetrahydroxyflavanol (Morin) an intense yellow-green fluorescence results, capable of indicating as little as 0·001 part per million.

Substances which give precipitates in alkaline solution must be removed by chemical means, but lithium, calcium, and zinc may interfere. Fluorescence due to lithium is approximately 1/1000 of that due to beryllium, and zinc and calcium may be prevented from affecting the result by the addition of cyanide and pyrophosphate respectively.

REAGENTS.

1. 2N *sodium hydroxide.*
2. *Sodium pyrophosphate.*
3. *Morin,* 0·2% *solution in acetone.*
4. *Sodium sulphide* 10% *in* 10% *sodium hydroxide.*
5. *Potassium cyanide,* 5% *solution in water.*

METHOD. The substance containing beryllium is treated with hydrogen sulphide in acid solution to remove heavy metals. The solution is then heated to remove hydrogen sulphide, made neutral, and treated with hot 10% sodium hydroxide/sodium sulphide solution to precipitate iron and manganese. This precipitate is dissolved and reprecipitated in order to extract beryllium, which may be retained by the precipitate. 5 ml. of the solution are then treated with 2 ml. of sodium pyrophosphate to avoid interference by calcium, and sodium hydroxide solution is added until the concentration is approximately 0·1N. 2 ml. of 5% potassium cyanide are then added to prevent interference by zinc, and then finally 0·1 ml. Morin solution is added. The solutions are then submitted to light from a mercury vapour source and the fluorescence is measured against standard beryllium solutions which have been treated in the same way.

(b) Fluorimetric Estimation of Riboflavin in Cereals and Other Products†

PRINCIPLES. The riboflavin is extracted with acid, separated by absorption on fuller's earth, eluted with acid-pyridine mixture, and measured fluorimetrically in blue activating light.

REAGENTS.

1. 0·25N *sulphuric acid.*
2. *Elution solution.* Mix 9 volumes of water, 5 volumes of pyridine, and 2 volumes of glacial acetic acid.

* Sandell, E. B., *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 674.
† Barton-Wright, E. C., and Booth, R. G., *Biochem. J.*, 1943, **37**, 25.

3. *Potassium permanganate, 4% solution.*

4. *Hydrogen peroxide, 10 volumes.*

5. *n-Butyl alcohol.*

METHOD. 20 g. of wheat (containing 20–50 mg. of riboflavin) are suspended in 100 ml. of 0·25N sulphuric acid in a 250 ml. flask. Two further flasks are put up, containing 20 g. of wheat, to which have been added 10 and 20 mg. of riboflavin respectively. The flasks are autoclaved for 15 minutes at 15 lb. steam pressure, cooled, and then the contents are adjusted to pH 5·0 using bromo-cresol green as an external indicator. After filtering, the clear liquids are transferred to three 50 ml. centrifuge tubes and 0·5 g. of fuller's earth is added to each and suspended in the solution by mechanical stirring. The tubes are next centrifuged, decanted, and the precipitate is washed with water and again centrifuged.

Removal of the adsorbed vitamin is then made by suspending the solid in 8 ml. of the elution solution and stirring mechanically for 5 minutes before centrifuging for a further 5 minutes at low speed. The eluate is then decanted into glass-stoppered cylinders and about 3 drops of permanganate are added to oxidise substances likely to interfere with the subsequent fluorescence measurement. After 1 minute, excess permanganate is destroyed by the controlled addition of a few drops of hydrogen peroxide.

To the clear liquid are now added 5 g. of anhydrous sodium sulphate and 20 ml. of butyl alcohol, and the mixture is brought to a temperature of 35°–40° C. It is shaken vigorously for 2 minutes and then is transferred to a centrifuge tube and spun for 5 minutes.

The clear alcohol layer is finally transferred to a fluorimeter cup and the fluorescence is measured in blue activating light using Wratten filter No. 47 and a Chance's orange-glass filter. After making the reading the riboflavin is destroyed by exposure to unscreened U.V. light for 1 hour. A second fluorescence reading is then made and the difference is taken.

Using the Spekker fluorimeter, the response is linear, and so a straight line may be drawn relating the fluorescence value of the three extracts to their riboflavin contents.

(C) TABLE OF FLUORIMETRIC METHODS

Substance	Reagent Used to Produce Fluorescence	Reference
	A. INORGANIC	
Aluminium.	Quercetin.	Davidov and Devekki, *Zavodskaya Lab.*, 1941, **10**, 134.
	Morin.	White, C., and Lowe, C., *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 229.

TABLE OF FLUORIMETRIC METHODS—*Continued*

Substance	Reagent Used to Produce Fluorescence	Reference
Gallium and Scandium.	8-Hydroxyquinoline (gallium complex in chloroform solution).	Sandell, E. B., *Ind. Eng. Chem.* (Anal. Edn.), 1941, **13**, 844.
Indium.	8-Hydroxyquinoline in chloroform.	Adapted from Moeller, T., *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15**, 270.
Uranium.	Sodium fluoride.	Neumann, W., Flemery, R., Coulson, A., and Glover, N., *J. Biol. Chem.*, 1948, **173**, 41.
Zinc.	Urobilin.	Lutz, R. E., *J. Ind. Hygiene*, 1925, **7**, 273.
Terbium. Cerium. Europium.	Aqueous solution.	Zaidel, A., Larionov, Y., and Filipov, A., *J. Gen. Chem. U.S.S.R.*, 1938, **8**, 943.

B.   ORGANIC

Chlorophyll.	Direct.	Kavanagh, F., *Ind. Eng. Chem.* (Anal. Edn.), 1941, **13**, 108.
Riboflavin = Lactoflavin = Vitamin B$_2$.	(i) Direct.	Cohen, F. H., *Rec. trav. Chem.*, 1935, **54**, 1330. Supplee, G., Bender, R., and Jensen, O., *Ind. Eng. Chem.* (Anal. Edn.), 1939, **11**, 495.
	(ii) Separation by absorption and elution with pyridine + acetic acid.	
Urobilin.	Zinc.	Lutz, R. E. (see under Zinc).
Vitamin B$_1$ (= Aneurin).	Oxidation to thiochrome.	Wokes, F., *Analyst*, 1942, **67**, 135 Compare *Analyst*, 1942, **67**, 15.

TABLE OF OTHER COLORIMETRIC METHODS

Substance	Principles	Reference
Alanine.	The reaction with ninhydrin produces acetaldehyde which is aspirated into bisulphite solution. This is treated with copper sulphate, sulphuric acid, and p-hydroxydiphenyl solution (see Lactic acid estimation, p. 324), and after incubation for 30 minutes the violet colour is read.	Alexander, B., and Seligman, A., *J. Biol. Chem.*, 1945, **159**, 9.
Paraldehyde.	The p-hydroxydiphenyl reaction (see Alanine) may be applied to paraldehyde.	Westfield, W., *J. Lab. Clin. Med.*, 1945, **30**, 1075.
Acyl phosphates.	Acyl phosphates react with hydroxylamine to produce hydroxamic acids which give a purple colour with ferric salts.	Seprian, F., and Tuttle, C., *J. Biol. Chem.*, 1945, **159**, 21.
Alcohol in blood.	Distillation of alcohol from a non-protein filtrate into acid dichromate. The green colour of the reduced chromic salt is measured against standards.	Gibson, J. G., and Blotner, H., *J. Biol. Chem.*, 1938, **126**, 551.
Fatty acids.	If fatty acids are methylated with diazomethane, then the hydroxamic acid reaction can be applied.	Hill, D., *Ind. Eng. Chem.* (Anal. Edn.), 1946, **18**, 317.
Formaldehyde.	Formaldehyde reacts with chromotropic acid in $15N$ sulphuric acid to produce a yellow colour.	McFadyen, J. *Biol. Chem.*, 1948, **158**, 107.
Formic acid.	Formic acid reduced with magnesium to formaldehyde which is measured by the chromotropic reaction (above).	Morton Grant, W., *Anal. Chem.*, 1948, **20**, 267.
Mannitol.	Oxidise with periodic acid to formaldehyde and then measure with chromotropic acid.	Corcoran, A., and Page, I., *J. Biol. Chem.*, 1947, **170**, 165.
Penicillin.	Penicillin reacts with N(1-naphthyl-4-azobenzene)-ethylene-diamine in organic solutions to produce a coloured compound which may be separated from excess reagent with aqueous sodium hydroxide.	Scudi, J., *J. Biol. Chem.*, 1946, **164**, 183.
Cysteine.	Reaction with brucine, glycine, and persulphate produces a blue colour.	Nakamura, H., and Binkley, F., *J. Biol. Chem.*, 1948, **173**, 407.
Urea.	In hot acid solution urea gives a red colour with isonitroso-propiophenone.	Archibald, R., *J. Biol. Chem.*, 1945, **157**, 507.
Vitamin A.	Vitamin A in oil is dissolved in chloroform and treated with 2 drops of concentrated HCl and 5 ml. of glycerol dichlorohydrin. Shake for 5 minutes and then read the stable blue-green colour after 30 minutes' standing.	Fernstein, L., *J. Biol. Chem.*, 1945, **159**, 569.
Vitamin K_3.	2-Methyl-1 : 4-naphthoquinone reacts with aromatic amines (e.g. aniline) to give a red colour.	Martinson, E., and Meerovitch, G., *Biokhimiya*, 1945, **10**, 258.

TABLE OF OTHER COLORIMETRIC METHODS—*Continued*

Substance	Principles	Reference
D.D.T.	Extract with mineral oil and react with xanthydrol, caustic potash, and pyridine to give a red colour.	Stiff, H., and Castilla, J., *Ind. Eng. Chem.* (Anal. Edn.), 1946, **18**, 316.
Cyanide.	Reaction with Chloramine-T to form cyanogen chloride, coupling with pyridine and subsequent formation of glutaconic aldehyde. Then reaction to give a blue colour with 1-phenyl-3-methyl-5-pyrazolone.	Epstein, J., *Anal. Chem.*, 1947, **19**, 272.
Silica in iron.	Silico-molybdate is reduced by ferrous iron to molybdenum blue. If oxalic acid is added then interference due to phosphorus, arsenic, and vanadium can be avoided.	Gentry, C., and Sherrington, L., *J. Soc. Chem. Ind.*, 1946, **157**, 507.
Titanium.	Reaction with di-sodium-1 : 2-dihydroxy-benzene-3 : 5-di-sulphonate to give a yellow colour. Iron in the ferrous condition does not interfere. Sensitivity: 1 in 200 million.	Yoe, J., and Armstrong, W., *Sci.*, 1945, **102**, 207.
Nickel.	The nickel-dimethylglyoxime complex may be dissolved in pyridine, and the red colour estimated directly.	Passamaneck, E., *Ind. Eng. Chem.* (Anal. Edn.), 1945, **17**, 257.
Sulphur dioxide.	After removal of interfering substances (e.g. H_2S) with mercuric chloride, SO_2 solution is treated with fuchsin in sulphuric acid and formaldehyde. A red colour is produced on heating.	Grant, W. M., *Anal. Chem.*, 1947, **19**, 345.

PART V

ELECTRO-CHEMICAL METHODS OF MICRO-ANALYSIS

By J. T. Stock

ELECTRO-CHEMICAL METHODS OF MICRO-ANALYSIS

GENERAL PRINCIPLES

METHODS of quantitative analysis which make use of the electro-chemical properties of the substance to be determined have been known for years and most of these have been applied with success on the micro scale. Some more recent techniques in micro-electro-chemical analysis, such as polarography, have, however, no counterpart in macro-chemistry.

Electro-chemical methods of analysis may be differentiated according to the basic principle involved. Techniques 1 to 4 below utilise the ability to accept or donate electrons which is possessed by the substance to be determined. Movement of charged particles in an electric field is the basis of procedures 5 and 6.

1. Potentiometry. The potential E_M of a single electrode of a metal in reversible equilibrium with its ions is given by the equation:

$$E_M = E_0 + \frac{RT}{zF} \log [\text{ion}]$$

or at ordinary temperature (17° C.)

$$E_M = E_0 + \frac{0 \cdot 058}{z} \log_{10} [\text{ion}]$$

where E_0 is the constant *standard electrode potential* for the metal involved, z is the valency of the ion, and [ion] is its concentration, or, more strictly, its activity in solution.

Consequently, the E.M.F. of an electrolytic cell comprising an *indicator electrode* and a *reference electrode* of fixed potential will vary as the *logarithm* of the concentration of the ion to be determined; the principle can be extended to include numerous non-metallic ions (e.g. hydrogen, halogens, etc.).

Potential measurements can thus be used:

(a) to determine ionic concentrations *directly*, as, for instance, in the determination of pH (see p. 411), or

(b) to indicate abrupt changes of ionic concentration, as in end-points of volumetric analysis (see pp. 413–415).

Since the *logarithm* of the ionic concentration is a significant factor, it follows that potential measurements are particularly sensitive in determining extremely low ionic concentrations.

Various elegant applications of the above basic principle have been used in micro-analysis, as exemplified on pp. 427–432.

2. Polarography. In this method, which has no counterpart on the macro scale, electrolysis is carried out between a dropping mercury electrode and a larger "quiet" electrode. A gradually increasing voltage is applied to the electrodes, the current passing through the solution being measured continuously or at short intervals. The current–voltage curve, or *polarogram*, which may thus be obtained often has a steeply rising portion or *wave*, followed by an almost flat portion in which the current changes little with further increase in applied voltage. The *position* of the wave along the voltage axis is characteristic of the *nature* of the substance being examined, while the *height* of the wave is frequently proportional to the *concentration* of the substance in the solution.

Amperometric titration (see pp. 473–483) is a comparatively recent development of polarography in which electrical measurements are made only as a means of detecting the end-point of a titration reaction.

3. Electro-deposition. Many metals, e.g. gold, copper, mercury, can be deposited quantitatively from solution by electrolysis of their solutions between electrodes of an inert material such as platinum, and thus collected and weighed. Since the deposition of a given substance does not take place until a certain characteristic potential—the *reduction potential*—has been exceeded, it is possible to perform various separations by controlling the applied voltage—or, more correctly, the potential of the electrode upon which the deposition is occurring. Usually deposition occurs upon the *cathode*, viz. the electrode at which electrons pass into the solution, metallic ions normally being reduced to the metallic state. Although *anodic deposition* is far less common, it has some important applications, such as the determination of lead as lead peroxide (see pp. 495–497).

Electro-deposition may be used directly as a method of gravimetric analysis, but alternatively it may be used as a method of separating particular components of complex mixtures prior to a colorimetric or other sensitive analytical procedure.

Electro-deposition methods can be used for quantitative analysis only when it is possible to expel an ion completely from solution, and is specially applicable to metals which stand low in the electric potential series. The application of these methods to non-metallic substances is limited.

4. Coulometric Analysis. It follows from Faraday's Laws of Electrolysis that the *quantity* of electricity necessary for effecting any quantitative reaction (e.g. reduction) of a substance can be used as a measure of the amount present in a solution. One technique of coulometric analysis

consists of electrolysing the solution until an initially added chemical indicator shows that the reaction is just complete. The total quantity of electricity used in bringing this about is measured by a coulometer connected in series with the electrolysis cell.

In an alternative technique described by Lingane,* reaction is effected at a stirred mercury electrode the potential of which is kept constant throughout the analysis. The completion of the process is indicated by the flow of current having fallen almost to zero. Though fundamentally not so accurate, this procedure is much more flexible, requires no additional indicators, and allows mixtures of electrolysable substances to be examined. Lingane's paper should be consulted for experimental details.

5. Conductometric analysis. The electrical conductivity of a solution is a function of the concentration and the mobility of all the ions present in it. Accordingly, conductivity can be used as a measure of ionic strength. For purely analytical purposes, absolute conductivity measurement is uncommon, though it may be used for the assessment of trace impurities, as in the determination of soluble solids carried over by steam from a boiler. However, *changes* in conductivity can often be used to follow the courses of chemical reactions, as, for example, in *conductometric titration*.

6. Electrophoresis. Measurements of the rates of migration of charged particles in an electric field are of considerable importance in the study of proteins, enzymes, and other biological products. In certain cases the material to be studied may be adsorbed upon quartz particles, the movement of which is observed by the ultramicroscope. In the more general method introduced by Tiselius, a sharp boundary between the protein or similar solution and the solvent is made. The motion of this boundary in the electric field is followed by refractive index observations.

Though simple in principle, electrophoretic methods usually require elaborate apparatus. Their limited application to a specialised field excludes them from detailed consideration in this book, but the following references may be consulted:

Abramson, Moyer, and Gorin, "Electrophoresis of Proteins" (Rheinhold, 1942); Cohn and Edsall, "Proteins, Amino-acids, Peptides" (Rheinhold, 1943).

* *J. Amer. Chem. Soc.*, 1945, **67**, 1916.

POTENTIOMETRIC TECHNIQUES

INTRODUCTION

As indicated on p. 407, measurement of the potential of an *indicator electrode* dipping into a solution can be used to determine the concentration in the solution of certain ions or to follow changes in their concentration. Unfortunately, the potential of a single electrode with respect to a solution cannot be measured directly; *two* electrodes, dipping into the same or into different solutions, may, however, be combined to form a voltaic cell, the E.M.F. of which is readily measurable. By using as a *reference electrode* an electrode/solution system of fixed potential, the potential of the indicator electrode may be obtained from the measured E.M.F. of the complete cell, as described below, provided that any internal liquid/liquid junction potentials are eliminated or at least kept constant.
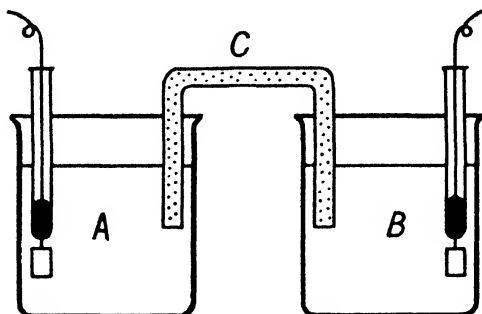
Fig. V.1. Combination of Two Electrode Systems to Form a Voltaic Cell.
A, test solution and indicator electrode; *B*, reference electrode system; *C*, salt bridge.

To minimise these latter "junction potentials" the solutions of the two electrode systems are usually connected by a "salt bridge" (see p. 422), often consisting of a saturated solution of potassium chloride or nitrate or of an agar jelly saturated with one of these salts. The arrangement is depicted schematically in fig. V.1.

Electrode potentials are measured on the arbitrary "hydrogen scale," on which the potential difference between a hydrogen electrode (see p. 416) saturated with gas at a pressure of 1 atmosphere and a solution of unit hydrogen ion activity is zero. Though this *normal hydrogen electrode* is the basis of our system of potential measurements, it is impracticable as a working reference electrode. It is therefore usually replaced with a more convenient and reproducible system, the potential of which upon the hydrogen scale has been determined. The usual reference electrodes (see p. 422) are positive to the normal hydrogen electrode.

DETERMINATION OF pH

1. Acidity and Alkalinity. The degree of acidity or alkalinity of the solution or medium is of vital importance in widely diverse fields of science. In analytical chemistry, for example, close control of acidity may be an essential factor in the attainment of reproducibility, while in bacteriology slight changes in acidity may have a far-reaching effect upon the rate of growth of micro-organisms. Biological fluids, e.g. blood, gastric juice, or pancreatic juice, usually have quite definite degrees of acidity or alkalinity, and determination of these may have considerable bearing upon clinical diagnosis.

Properties expressed by the vague terms "acid," "neutral," or "alkaline" are controlled by a single quantity, the concentration* of hydrogen ions in the solution.† The concentration to be considered ranges from about 10^{-14} to 10 gram-ions of hydrogen per litre, alkaline solutions having low and acid solutions high concentrations. Concentration within this great range is best expressed logarithmically, as was proposed by Sörenson in 1909. He introduced the term *hydrogen ion exponent*, designated by the symbol pH and given by

$$p\text{H}=-\log_{10}[\text{H}^+]$$

where $[\text{H}^+]$ is the concentration* of hydrogen ions in the solution.

2. The Hydrogen Electrode. When saturated with gaseous hydrogen, a piece of platinum coated with platinum black and dipping into a solution containing hydrogen ions acts potentiometrically as if it were composed entirely of hydrogen and acquires a potential given by the general equation

$$E_H = E_0 + \frac{0.058}{z}\log_{10}[\text{ion}]\ (\text{at }17°\text{ C.})$$

By definition, however, the standard electrode potential of hydrogen is zero, so that the equation reduces to

$$E_H = 0.058\log_{10}[\text{H}^+]$$

or since $-\log_{10}[\text{H}^+]$ may be written as pH

$$E_H = -0.058\ p\text{H}.$$

Thus the pH of a solution may be calculated directly from the potential acquired by a *hydrogen electrode* dipping into it.

Though being the ultimate standard to which other electrodes are referred and exhibiting no error in solutions of high pH, the hydrogen electrode has many practical disadvantages. Of these, the necessity for a supply of pure hydrogen, the sluggishness in attaining a steady potential, and the ease of

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* Strictly, the hydrogen ion *activity*, or effective concentration. [See, however, Glasstone, "The Electrochemistry of Solutions" (Methuen, London, 1937), p. 189, footnote.] In very dilute solutions the concentration and activity become almost identical.

† Hydrogen ions do not exist in solution as such, but are in fact hydrated: $\text{H}^+ + \text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+$.

"poisoning" may be mentioned.   Hence it is not normally employed when other electrode systems are applicable.

Various forms of micro-hydrogen electrodes are described on pp. 416–417.

3. The Quinhydrone Electrode. The oxidation-reduction potentials of many chemical systems depend upon the pH of the solution, thus affording a possible means of determining pH without the use of hydrogen.   Biilman* showed that the molecular compound quinhydrone could be used for this purpose, if sufficient were added to saturate the solution to be examined. On inserting an inert electrode, usually of bright platinum or gold, it acquires a potential E_Q given by

$$E_Q = 0 \cdot 7051 - 0 \cdot 058 \; p\text{H (at } 17° \text{ C.)}$$

thus permitting the pH of the solution to be calculated from the value of E_Q.
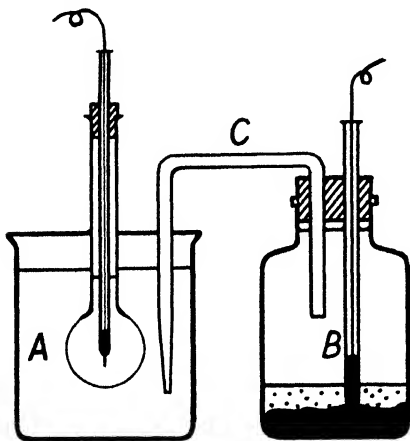
Fig. V.2.   Determination of pH by the Glass Electrode.

A, bulb of glass electrode; B, reference electrode system; C, salt bridge.

The quinhydrone electrode possesses the supreme advantage of simplicity and its possibilities have been considerably explored, including applications to the micro scale (see p. 417).   Unfortunately it cannot be used in solutions of pH greater than about 8.   Though not easily "poisoned," it gives rise to errors in the presence of certain proteins and salts.   In general, oxidising or reducing agents may not be present in the solution, while for accurate work a correction for dissolved salts ("salt error") is necessary.

4. The Glass Electrode. A most important method of pH determination was developed from the discovery that the potential difference between a suitable glass surface and a solution in contact with it varies with the pH of the solution.†   The arrangement is depicted in fig. V.2.   Within the thin bulb A of special glass dipping into the solution to be examined is an electrode/solution system of fixed potential, such as a silver/silver chloride electrode immersed in $0 \cdot 1N$ hydrochloric acid (see p. 422).   The potential of the glass electrode is determined by connecting the test solution to a reference electrode in the usual way, and the pH of the solution is calculated from

$$E_G = E_O - 0 \cdot 058 \; p\text{H (at } 17° \text{ C.)}.$$

* *Ann. Chim.*, 1921, **15** (a), 109.
† Haber and Klemensiewicz, *Z. physik. Chem.*, 1909, **67**, 385.

The value of E_0 varies from one electrode to another, and in practice the system is calibrated with a solution of known pH ("buffer solution").

Advantages possessed by the glass electrode are: (1) hydrogen, quinhydrone, or other additives are not required; (2) oxidising, reducing, or capillary-active agents do not affect the potential and the latter is attained rapidly; (3) very small amounts of liquid may be examined (see p. 419); (4) it can be used to measure the pH of unbuffered solutions.

On the other hand, the thin glass membrane is necessarily fragile and is of high electrical resistance; to measure its potential a valve electrometer (see p. 426) is normally required. Further, the simple equation given above does not hold in strongly acid or in strongly alkaline solutions, so that special calibration is necessary for use in such solutions.

The numerous advantages of the glass electrode system have, however, resulted in the production of numerous commercial instruments[*] in which these inherent disadvantages are minimised.

5. Other Electrode Systems. Since the potentials of certain metal/metal oxide electrodes change with pH in the same way as does that of the hydrogen electrode, these electrodes may also be used for pH determination. Most important is the antimony/antimony oxide electrode, while in strongly alkaline solutions the mercury/mercuric oxide and silver/silver oxide electrodes may be used. Discussion of this class of electrode is outside the scope of this book, but the following references may be consulted:

Clark, "The Determination of Hydrogen Ions" (Biallière, Tindall and Cox, London, 1928); Glasstone, "The Electrochemistry of Solutions" (Methuen, London, 1937); Furman, *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 367.

6. Choice of Electrodes. Owing to its simplicity, the quinhydrone electrode is to be specially recommended for examining solutions of pH less than about 8. Its limitations in the presence of oxidising or reducing agents, of certain proteins, etc., should, however, be borne in mind.

The glass electrode does not suffer from these limitations and is to be recommended when the necessary electrical equipment is available. For certain biological purposes, as for example measurement of the pH of living tissue, its advantages are obvious.

POTENTIOMETRIC TITRATION METHODS

1. Acid-Base Titration. During the neutralisation of an acid by a base, or *vice versa*, the pH of the solution changes most rapidly in the neighbourhood of the equivalence point. Hence the latter may frequently be determined by plotting the pH of the solution against the volume of reagent

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[*] See, for example, List 108A, Cambridge Instrument Company, Ltd.; Marconi Instruments, Ltd.; Dole, "Glass Electrode" (Wiley, New York, 1941), p. 41, footnote.

added.   In certain cases, such as in the titration of a strong acid with a strong base (or the reverse), the pH change is very large, as shown by fig. V.3; here the equivalence or end-point may be observed without difficulty.

For accurate work or when the pH change is less marked, the end-point is determined by adding the reagent in small, definite amounts ΔV (e.g. 1 drop) and measuring the change in potential ΔE thus produced.   By plotting $\Delta E/\Delta V$ against the volume of reagent added a curve of the type shown in fig. V.3, b is obtained, the maximum upon the curve indicating the end-point volume.

Suitable forms of any of the hydrogen-ion electrodes may be used for acid-base titration.   When using quinhydrone it is advisable to add it to the acid solution and to titrate the latter with the base.
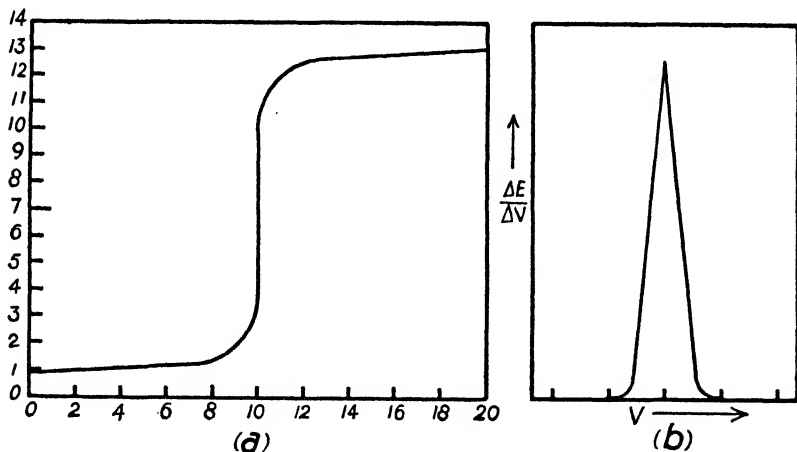
Fig. V.3.
(a) pH Change During Titration.
10 ml. of $0.1N$ hydrochloric acid titrated with $0.1N$ sodium hydroxide solution.
(b) Determination of Titration End-point.

Electrical measurements are made here only to detect the end-point and do not enter into the calculation of the analytical result.   Accordingly, the E.M.F. of the cell combination, which is directly related to the pH, may be plotted instead of the latter.   Analogous remarks apply to the other classes of reactions discussed below.

2. Precipitation Titrations. In the precipitation of an ion X from solution by addition of a suitable reagent, as for example in the titration of a soluble chloride with silver nitrate solution, the concentration of X in the solution will obviously change most rapidly in the region of the end-point.   The potential of an indicator electrode responsive to the concentration of X will undergo a like change; by plotting the potential against the volume of reagent added, a curve similar to that of fig. V.3, a may be obtained.   From

this, the end-point may be observed directly, or it may be obtained by the "increment" method of fig. V.3, *b*.

Where the solution contains more than one kind of ion capable of being precipitated by the reagent, a titration curve showing successive potential jumps is sometimes obtained, each corresponding to the completion of precipitation of one kind of ion.

3. Oxidation-Reduction Titrations. The potential of an unattackable electrode, such as a plate of bright platinum, immersed in an oxidation-reduction system (e.g. one containing both ferrous and ferric ions) depends upon the relative amounts of oxidised and of reduced form present in the solution. Considering the general reaction

$$\text{Oxidised form} + z \text{ electrons} \rightleftharpoons \text{Reduced form}$$

the potential E_1 acquired by the indicator electrode is given by

$$E_1 = E_0 + \frac{0\cdot058}{z} \log_{10} \frac{[Ox]}{[Red]} \text{ (at 17° C.)}$$

where E_0 is the standard oxidation potential of the system and z the number of electrons taking part in the reaction, while (Ox) and (Red) are the respective concentrations* of oxidised and reduced forms (compare p. 137).

The potential of the immersed electrode is thus controlled by the *ratio* of these concentrations. During the reduction of an oxidising agent or the oxidation of a reducing agent this ratio, and hence the potential, changes most rapidly in the region of the end-point of the reaction. Accordingly, titrations involving such reactions, such as the titration of ferrous iron with dichromate or permanganate, may be followed potentiometrically and yield titration curves characterised by a sudden change of potential at the end-point.

An example of this class of titration is given on p. 430.

4. Bimetallic Electrode Systems. In potentiometric titration, simplified electrode systems may often replace the classical cell arrangement so far considered.

If two electrodes of dissimilar materials, one of which responds to changes in an oxidation-reduction system while the other does not, are inserted in such a system, the E.M.F. developed by the electrode pair will change during titration. The "indifferent" electrode (e.g. one of palladium) thus serves as an *internal* reference electrode to the normally acting indicator electrode (e.g. one of platinum). Bimetallic systems may also be used for acid-base and other titrations.

By applying a small "polarising" E.M.F., a pair of *similar* electrodes (e.g. of platinum) may be used in place of dissimilar ones. For instance, the "dead stop" method† has attracted considerable attention. As shown

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* More strictly, the activities.
† Foulk and Bawden, *J. Amer. Chem. Soc.*, 1926, **48**, 2045. For bibliography to the end of 1946 see Stock, *Metallurgia*, 1948, **87**, 220.

in fig. V.4, this arrangement is one of extreme simplicity.   The end-point is characterised by a sudden movement of the galvanometer needle from zero to a large deflection or *vice versa*.   Though usually classified as a "potentiometric method," this titration is carried out at fixed voltage, a greatly increased flow of *current* indicating the end-point.   A valve system which supplies the polarising E.M.F. and serves to detect the end-point may be used in place of a galvanometer (see p. 427).   For an example of the use of this method see p. 431.

Several other methods of end-point detection have been devised.*

APPARATUS AND GENERAL TECHNIQUE

1. Electrodes for pH Determination

(*a*) *Hydrogen Electrodes*. Coating with "platinum black" of the platinum
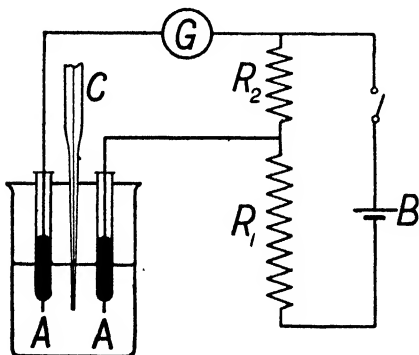
Fig. V.4.

Apparatus for "Dead Stop" Titration.
A, A, platinum electrodes; *B*, 1·5 volt cell; *C*, burette; *G*, galvanometer; R_1, R_2, resistances.

wire or foil in contact with the solution is an important operation, and is conducted as follows.   Having been thoroughly cleaned by successive treatment with (i) alcoholic sodium hydroxide, (ii) water, (iii) chromic acid solution, and (iv) water, the electrode is immersed in a 3% solution of platinic chloride acidified wi+h hydrochloric acid and connected to the negative terminal of a 2 volt accumulator.   A platinum wire serving as anode, electrolysis is carried out until the brightness of the platinum disappears.   After rapidly washing the electrodes, the platinic chloride solution is replaced by one of 10% sulphuric acid.   The electrolysis is continued, increasing the voltage to 4, until the deposit is thoroughly charged with hydrogen and gas streams uniformly from the surface.   If the deposit is not uniform, it should be removed before recoating by making the electrode the anode in 1 : 1 hydrochloric acid.   When not in use, the electrode should be immersed in distilled water to prevent the deposit from becoming dry.

The *Lockwood electrode* (fig. V.5, *a*) is suitable for examining samples of about 1 ml. in volume.†   A wire-form platinised platinum electrode,

* See, for example, Kolthoff and Furman, "Potentiometric Titrations" (2nd edn., Wiley, New York, 1931); Furman, *Ind. Eng. Chem.* (Anal. Edn.), 1930, **2**, 213; ibid., 1942, **14**, 367.

† Lockwood, *J. Soc. Chem. Ind.*, 1935, **54**, 295.

pointed at the lower end, is used, so that the hydrogen bubbles are pierced and in rising come in contact with the whole length of the electrode. A calomel reference electrode (see p. 422) with a fine outlet dips into the open tube and enables the cell to be completed.

For volumes of liquid of from 0·005–0·06 ml. the electrode shown in fig. V.5, *b* may be used.* The upper end of the 1 mm. bore tube is expanded into a little cup *A*, in which the sample is placed. The platinised platinum electrode is a 3 mm. disc formed at the end of a piece of stout platinum wire, the latter being sealed through the side of the cup as shown. Connection to the reference electrode *B* is made by a short length of cotton or similar thread. Hydrogen introduced at the lower end to cause bubbles to form at the rate of 30 per minute raises the liquid into contact with the electrode.
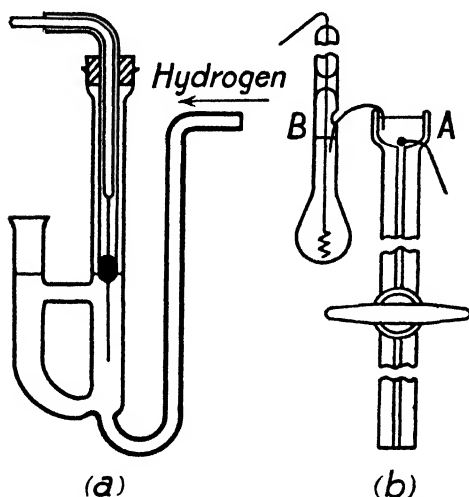
Fig. V.5. (*a*) The Lockwood Micro-hydrogen Electrode. (*b*) The Frediani Micro-hydrogen Electrode.
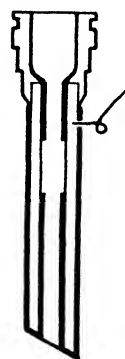
Fig. V.6. The Mikawa Micro-quinhydrone Electrode.

(*b*) *Quinhydrone Electrodes.* The *Mikawa electrode* (fig. V.6) allows samples of liquid as small as 0·01 ml. to be examined.† It consists of a thin gold tube of about 1 mm. bore. With the exception of the middle third of the bore, which is gold-plated, the electrode is coated with bakelite varnish. A drop of the liquid to be tested and a little finely powdered quinhydrone are placed on a paraffined watch-glass, and the drop is caused to roll until well covered with quinhydrone. By operation of a syringe attached by means of the rubber nipple the sample is drawn into the electrode. For

* Frediani, *Ind. Eng. Chem.* (Anal. Edn.), 1939, **11**, 53. For other micro-hydrogen electrodes, see Dorfmann, *Protoplasma*, 1936, **25**, 465; Lobering, *Z. anal. Chem.*, 1935, **103**, 180.

† *Biochem. J.*, 1933, **27**, 1829.

E.M.F. measurement the tip of the latter is then thrust into agar/potassium chloride gel (see p. 422) making contact with the reference electrode.

In the apparatus shown in fig. V.7* the sample is placed upon the concave extremity of the vertical tube rising from the base of the chamber. A small hole in the wall of the tube enables air to be swept out by a current of nitrogen when desired. A gold-plated platinum wire type electrode is used, which
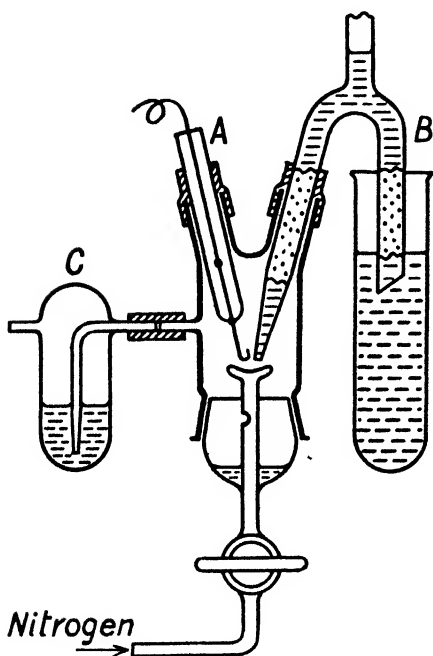
Fig. V.7. The Fuhrmann Apparatus.
A, quinhydrone electrode; B, salt bridge; C, trap.
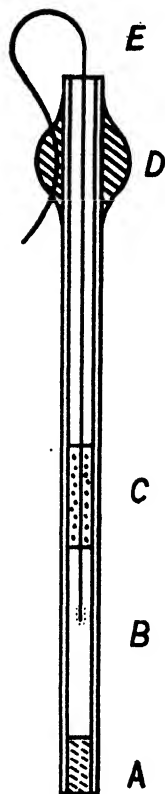
Fig. V.8. Apparatus for Examining Samples Collected Under Oil.
A, agar/potassium chloride plug; B, sample; C, oil; D, cement; E, electrode.

before insertion into the cell may be coated with quinhydrone, thus introducing the latter into the sample.

Biological fluids and other liquids rapidly affected by the atmosphere may be examined in complete absence of air in the device shown in fig. V.8, and the sample may be collected directly under sterile conditions.† The

* Fuhrmann, *Mikrochemie*, Molisch Festschrift, 1936, 130.
† Pierce, *J. Biol. Chem.*, 1937, **117**, 651.

electrode, which is not inserted in the 10 cm. length of 0·33 mm. bore quartz capillary until electrical measurements are to be made, is a length of 36-gauge platinum iridium wire the tip of which is coated with quinhydrone.

To examine, for example, cerebro-spinal fluid, the procedure is as follows. Immediately before use the capillary is sterilised in an alcohol flame. One end is then dipped into sterile oil, so that a 5 mm. column of the latter is picked up. This end is now thrust into the shoulder of the needle making the lumbar puncture, when the issuing fluid follows the oil up the capillary. When a 1 cm. length has entered, the capillary is withdrawn, thrust through a previously prepared disc of agar/potassium chloride gel, thus isolating the sample from the air and enabling the capillary to be carried in an upright position.

The tip of the platinum-iridium wire is dipped into a sludge of quinhydrone and acetone and, after the solvent has evaporated, is pushed down the capillary through the oil layer until half-way into the column of fluid. The wire is then bent back and secured by plasticine or de Khotinsky cement as shown. Connection to the reference electrode is made by the agar/potassium chloride plug. In a similar manner, portions of liquid stored beneath oil may be withdrawn and examined.

Various other micro-quinhydrone electrodes have been designed.*

(c) *Glass Electrodes*. A thin-walled bulb blown at the end of a piece of glass tubing constitutes the simplest form of glass electrode (a glass of special composition is employed). The bulb is usually filled with $0·1N$ hydrochloric acid, into which dips a silver/silver chloride electrode (see p. 422), to which electrical connection is made. A commercially-available electrode of the bulb type is shown in fig. V.9.

Alternatively, the thin membrane of special glass may be in the form of a diaphragm sealed to the end of a supporting tube, as shown in fig. V.10.† In a third form, a thin-walled capillary constitutes the membrane (*vide infra*).

For details of the construction of these and of other designs and for precautions as to their use the reader is referred to the monograph of Dole.‡

Convenient glass electrode systems for handling very small volumes of liquid are illustrated in figs. V.11 to V.13.

The capacity of the electrode chamber of the *Stadie cell* (fig. V.11) is only about 0·2 ml.§ An additional bulb above the chamber may be filled

* See, for example, Vodret, *Rend. seminario Facolta sci. univ. Cagliari*, 1933, **3**, No. 2, 55; *Chimie et industrie*, 1933, **31**, 1339; La Mer and Armbruster, *J. Amer. Chem. Soc.*, 1935, **57**, 1510; La Mer and Rule, ibid., 1938, **60**, 1974; Kranz, Carr, and Musser, *Science*, 1937, **85**, 127; Itano, *Comp. rend. trav. lab. Carlsberg. ser. chimique*, 1938, **22**, 235.

† MacInnes and Dole, *Ind. Eng. Chem.* (Anal. Edn.), 1929, **1**, 57.

‡ "The Glass Electrode" (Wiley, New York, 1941).

§ Stadie, O'Brien, and Laug, *J. Biol. Chem.*, 1931, **91**, 243; List 108A, Cambridge Instrument Co., Ltd., p. 13.

with oil to prevent contact of the sample with air.   The whole is contained in a water-jacket to stabilise the temperature.

In the *apparatus of MacInnes and Dole* shown in fig. V.12,* the calomel reference electrode A is provided with a reservoir B containing saturated
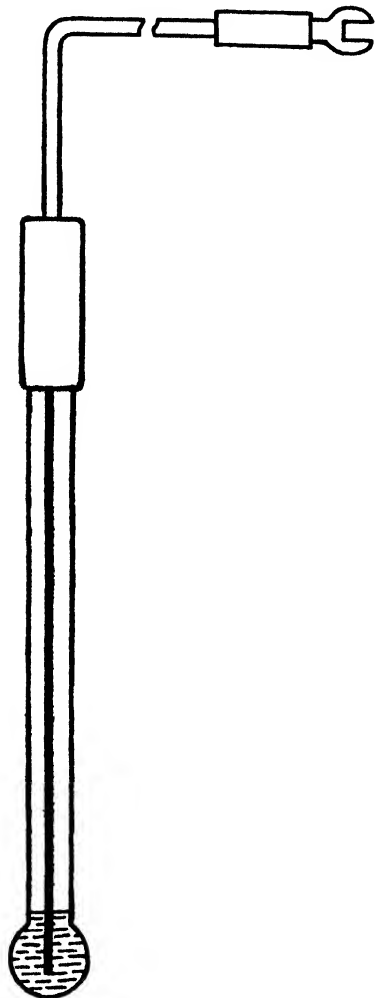
Fig. V.9.   The "Cambridge" Glass Electrode.
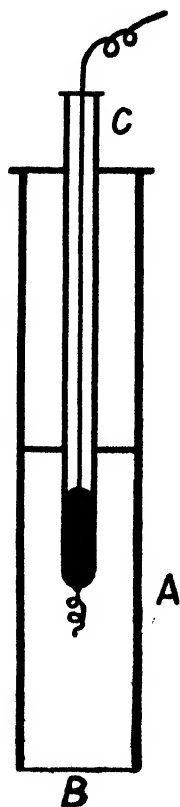
Fig. V.10.   Diaphragm-type Glass Electrode.

A, supporting tube; B, thin membrane; C,   silver/silver   chloride electrode.

potassium chloride solution.   By compressing a short length of rubber tubing attached to side-tube C, capillary outlet D may be swept out with potassium chloride solution, the excess being removed by filter-paper.   A drop of sample is placed on D and, by slightly loosening the clamp, is sucked

* *J. Gen. Physiol.*, 1929, **12**, 805.

into the capillary for about 1–2 mm. A second drop of the sample is
placed on D after again wiping the latter. Having fitted the shield F,
the diaphragm glass electrode E is lowered mechanically until the thin
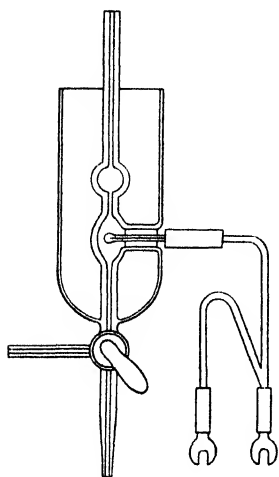
Fig. V.11.   The Stadie Apparatus.
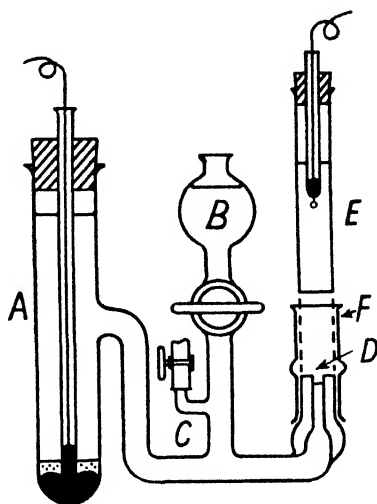
Fig. V.12.   Apparatus for Examination of
a Drop of Liquid.
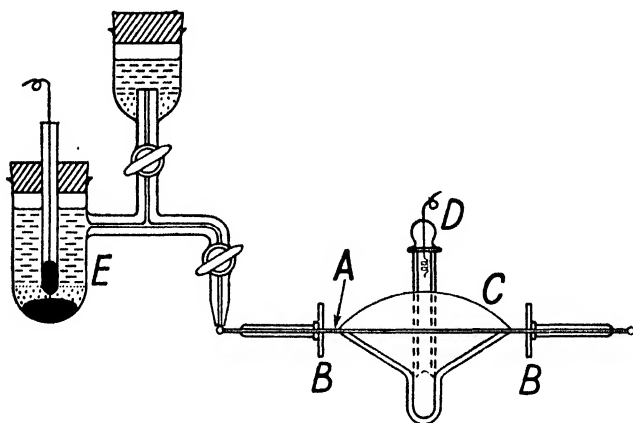
Fig. V.13.   Capillary-type Glass Electrode System.
A, capillary containing sample; B, B, insulating discs; C, fan-shaped funnel; D, silver/silver
chloride electrode; E, calomel electrode.

membrane makes contact with the drop of sample, when the E.M.F. is
quickly measured.

The use of glass electrode assemblies in which the membrane is in the
form of a capillary permits very small samples to be examined. In the

apparatus shown in fig. V.13,* the capillary containing the sample is supported by insulating discs so that the middle portion is immersed in 0·1N hydrochloric acid, with which the fan-shaped funnel is filled to capacity and into which dips a silver/silver chloride electrode. The assembly is then raised until junction is made with the specially designed saturated calomel electrode, when the E.M.F. of the cell is measured.

2. Reference Electrodes

The *saturated calomel electrode* is the commonest of these, and has a potential of $+0.2509$ volt at $17°$. A simple form shown in fig. V.14 is particularly suitable for potentiometric titration. A pool of mercury, electrical contact to which may be made as shown at (a), is covered with a layer of calomel and finely divided mercury wetted with saturated potassium chloride solution. The electrode vessel is filled with the same solution which has been saturated with calomel. The interchangeable syphon tube is filled with agar/saturated potassium chloride gel and serves as a "salt bridge."

For special purposes (for example, see p. 428) the calomel electrode may be prepared with potassium chloride solution of other concentrations. The potential is then higher than that of the saturated electrode.

The *silver/silver chloride* electrode consists of a spiral of silver wire coated anodically with silver chloride and dipping into a solution containing chloride ions (for example, 0·1N hydrochloric acid or saturated potassium chloride solution). A micro-electrode of this type is shown at A in fig. V.16.† To prevent escape of liquid, the lower end is closed by a tiny sintered glass plate.

Fig. V.14.
Bottle-form Calomel Electrode.

(a)

The *Schwarz reference electrode* for drop-scale titration is described on p. 424.

To enable contact to be made with the solution to be examined, a *salt bridge*, usually filled with a solution or gel saturated with potassium chloride, is employed. When using the saturated calomel electrode, one end of such a salt bridge makes direct contact with the solution within the electrode vessel. The other end, which is suitably drawn out, dips into the test solution. Interchangeable salt bridges of the type shown in fig. V.1 (p. 410) are filled with a hot solution prepared by gently heating 3 g. of agar and 30 g. of potassium chloride with 100 ml. of water until clear and

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* Claff and Swenson, *J. Biol. Chem.*, 1944, **152**, 519.
† Krumholz, *Mikrochemie ver. Mikrochimica. Acta*, 1938, **25**, 244.

free from air bubbles.   When the solution in the salt bridges has set to a gel, the latter are stored in saturated potassium chloride solution until required.   If it is undesirable for the chloride-containing salt bridge to contact directly the test solution (as, for example, when the latter contains silver salts), an intermediate vessel and a second salt bridge filled with potassium or ammonium nitrate solution or gel may be used, as illustrated in fig. V.7 (p. 418).

Another form of salt bridge is a capillary integral with the apparatus and filled from a reservoir with saturated potassium chloride solution (see, for example, fig. V.13, p. 421).   Whereas a gel-filled bridge is normally used once only, liquid-filled bridges need only to be flushed out between determinations with a few drops of potassium chloride solution from the reservoir.

In certain cases (see, for example, fig. V.5, b, p. 407) a thread of cotton or similar material moistened with saturated potassium chloride solution is a useful form of salt bridge.

3. Titration Assembly

The congestion caused by the necessary presence in the micro-titration vessel of burette tip, indicator electrode, and reference electrode or salt bridge renders difficult the essential operation of stirring.   In this connection the short-stroke vacuum-operated stirrer shown in fig. V.15 is often useful,* since the fine, flexible extremities of the interchangeable stirrer heads are readily bent to any shape and will not damage burette or electrodes should accidental contact occur.

In the absence of volatile substances, stirring may be effected by bubbling a current of nitrogen or other suitable gas through the solution (see Volumetric Section, p. 159).   Another alternative is to cause the titration vessel to rotate† or to vibrate.‡   The former method is illustrated in fig. V.16, while the latter is described in the following section.

For acid-base titration with a bulb-type glass electrode the simple micro-titration vessel described by Catch, Cook, and Kitchener§ permits effective mixing by manual agitation.

4. Potentiometric Titration of a Single Drop of Liquid‖

In this technique the drop of liquid is suspended from a ring-shaped indicator electrode, which thus also serves as a solution-container.   The

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* Fill and Stock, *Analyst*, 1944, **69**, 212.
† Raspopov and Finkelshtein, *Zavodskaya Lab.*, 1936, **5**, 353; Krumholz, *Mikrochemie ver. Mikrochim. Acta*, 1938, **25**, 244.
‡ Schwarz, *Mikrochemie*, 1933, **13**, 6; ibid., 1935, **18**, 106.
§ *J.C.S.*, 1945, 319.
‖ Schwarz, *Mikrochemie*, 1933, **13**, 6.   See also Zürcher and Hoepe, *Helv. Chim. Acta*, 1938, **21**, 1272.

apparatus is shown in fig. V.17. For argentometric titration, the indicator electrode is of 0·3 mm. silver wire, the ring having an external diameter of 4 mm. A platinum electrode A of similar dimensions is used for oxidation-reduction work. The extremity of the indicator electrode is attached by way of an intervening insulator B to the reed of a loud-speaker unit C. The latter is energised by the A.C. mains, a variable resistance permitting the magnitude of the vibrations of the reed to be controlled. Effective stirring during titration is thus obtained.
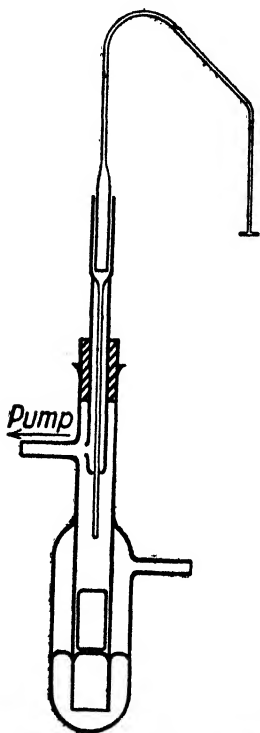
Fig. V.15. Vacuum-operated Micro-stirrer with Interchangeable Heads.
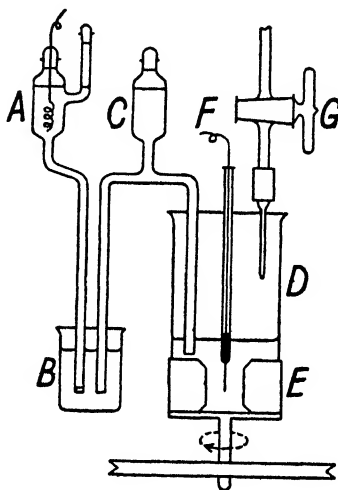
Fig. V.16. Stirring by Rotation of Titration Vessel.

A, reference electrode; B, intermediate solution; C, salt bridge; D, micro-beaker containing solution to be titrated; E, rotating holder; F, indicator electrode; G, micro-burette.

The tips of the micro-burette D and of the reference electrode E dip into the drop of solution. With a capacity of about 0·01 ml., the micro-burette can be read to 0·00001 ml. A micro-burette of simple construction was developed by Schwarz for this purpose,* others of requisite capacity (see pp. 146–154) may of course be used. Reference electrode E is constructed from 0·8 mm. bore capillary. The lower end is drawn out until about 0·3 mm. in outside diameter and of 0·05 mm. bore. This portion is about 4 cm. long. A short length of platinum wire, to the inner end of which is

* *Mikrochemie*, 1933, **13**, 1, 11.

welded a small piece (*ca.* 2 mg.) of pure silver, is sealed through the upper end of the capillary. By repeated evacuation and re-admission of air, the wider portion of the vessel thus formed is filled with 0·001N silver nitrate solution, while the fine portion is filled with 0·1N potassium nitrate solution, which serves as a salt bridge. For titrations in which the platinum ring electrode is used, the silver tip is omitted from the platinum wire, while a filling of 0·001N sodium thiosulphate replaces that of silver nitrate.

To avoid polarising the tiny electrodes, the current drawn from the system should be vanishingly small. Originally, a Lindemann electrometer, the needle of which was observed by means of a reading microscope, was used; but a valve electrometer (see p. 426) is simpler to read and to operate.

The general procedure in performing a titration is as follows. A micro-drop (0·02 ml.) of water is placed upon the ring electrode, and, without touching the latter, the tip of the reference electrode is inserted. The solution to be titrated is then added, usually from a micro-pipette. Having inserted the tip of the micro-burette containing the reagent, stirring is commenced and the titration at once proceeded with, noting the electrometer reading after each addition of reagent. It is obvious that the equivalent strength of the reagent should be greater than that of the solution to be titrated; otherwise, the drop may grow until it reaches breaking-point.

An example of the use of this technique is given on p. 430.
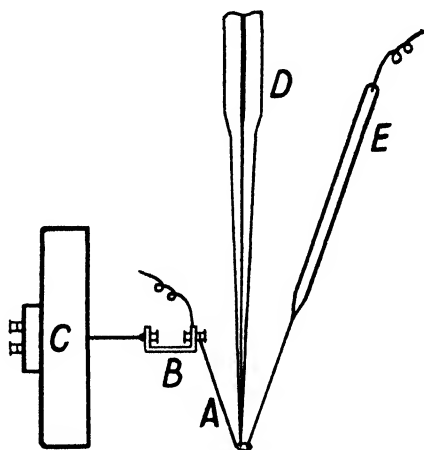
Fig. V.17.
Apparatus for Drop-scale Titration.

5. Measurement of Electromotive Force

In the measurement of the electromotive force no appreciable current may be drawn from the cell system during the operation; otherwise polarisation effects cause erroneous results. Accordingly a potentiometer method, in which the E.M.F. to be measured is balanced by an opposed but known E.M.F., is usually employed. In practice, a dial-type potentiometer is more convenient than a simple slidewire.

When using this method care must be taken to see that the tapping key is depressed momentarily to minimise the current drawn from the cell system under examination while the point of balance is being found.

The simple potentiometer method is unsuitable for cell systems of very high internal resistance, as, for example, most of those involving the use of the glass electrode. In such cases a Lindemann or similar electrometer or,

more conveniently, a thermionic valve electrometer must be employed. Since the current drawn from an electrolytic cell by an electrometer is negligibly small, the cell system may be permanently connected to the electrometer during the entire experiment, instead of momentarily as in potentiometer technique. This has the advantage that the E.M.F. may then be observed continuously; if a change occurs, as in potentiometric titration, no manipulation of controls is necessary. In a valve electrometer system, the E.M.F. change may be caused to operate an electron-ray tuning indicator.

Numerous designs of valve electrometer have been described* and various commercial instruments are available.† Illustrative of the simpler type of apparatus especially suited to potentiometric titration is that of Garman and Droz;‡ the circuit of this is shown in fig. V.18.
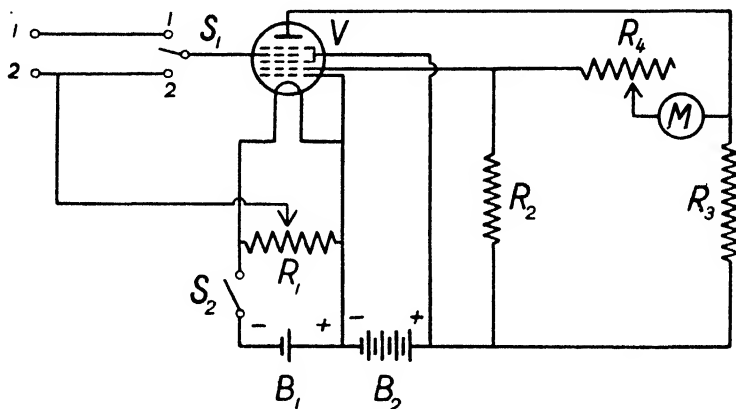
Fig. V.18. Continuous-reading Electrometer for Titration.
R_1, 1,000 ohms; R_2, 2,000 ohms; R_3, 2,500 ohms; R_4, 50,000 ohms; S_1, S_2, switches; M, meter, 0-50 microamperes; B_1, battery, 1·5 volt; B_2, battery, 45 volts; V, pentagrid valve, No. 1A7G.

Operation is as follows. The unit is switched on at lowest sensitivity and left to warm up for a few minutes. Switch S_1 is then moved to position 1, the cell-system being connected so that the electrode which will become more negative as the titration proceeds is attached to terminal 1. Potentiometer R_1 is adjusted until the microammeter reads zero, when the sensitivity is increased to suit the change in E.M.F. expected. The titration is then followed merely by observing the microammeter reading.

If the cell-system develops an E.M.F. initially too high to render zero-setting possible, connections to the electrodes are reversed and R_1 is adjusted until a *maximum* reading is obtained. The latter should then *decrease* as

* See, for example, Dole, "The Glass Electrode" (Wiley, New York, 1941), pp. 41–63.
† See, for example, List 108A, Cambridge Instrument Co., Ltd.; List No. 30.12, Marconi Instruments, Ltd.; List No. A/1, Baird and Tatlock, Ltd.
‡ Garman and Droz, *Ind. Eng. Chem.* (Anal. Edn.), 1939, **11**, 398.

the titration proceeds.   When this method fails, an auxiliary source of constant E.M.F. (e.g. a Weston standard cell) should be connected in opposition to the cell-system, thus reducing the total E.M.F. to a workable value.

The polarising E.M.F. required in the "dead stop" method (see pp. 415 and 431) may be provided by a valve system which also serves for end-point detection.*

REPRESENTATIVE EXAMPLES OF POTENTIOMETRIC TITRATION

The first example, the determination of boron as boric acid, has been selected to show how small changes in pH can be detected potentiometrically. Though a milligram or less of boron may be present in a volume of solution of several hundred millilitres, accurate titration is possible.   None of the special manipulations usually associated with micro-chemistry are involved, while a simple modification of the electrode system enables the potentiometer to be dispensed with.

The second example is of an oxidation-reduction titration which, in contrast to the above, illustrates the technique of titrating extremely small volumes of solution.   Quantities of a few micrograms of material may thus be determined.

The third example illustrates the use of bimetallic electrode systems, which greatly simplify procedures in which the rigid exclusion of extraneous moisture is essential, as, for instance, in the determination of small quantities of water by the Karl Fischer method.   The "dead stop" technique is employed, so that no electrical manipulations or measurements are required, the end-point of the titration being indicated by a sudden change either in the deflection of a galvanometer or in the shadow area of a "magic eye" indicator.

Example 1: The Determination of Boron

PRINCIPLES. If a polyhydric alcohol such as glycerol or mannitol is added to an unbuffered solution of boric acid at an electrically measured pH (approximately 7), the acidity of the boric acid is enhanced and the pH of the solution falls.   By titrating with standard alkali until the pH of the solution reaches exactly its original value the amount of boric acid present can be determined.

If a reference electrode which has the same potential as that acquired by a quinhydrone electrode at the chosen pH value is employed, then the

* See, for example, Serfass, *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 536; McKinney and Hall, ibid., 1943, **15**, 460; "Mullard-B.T.L. Potentiometric Titration Apparatus," Baird and Tatlock, Ltd., London.

titration may be followed merely by observing the deflection of a
galvanometer connected to the electrodes.*   At the end-point the deflection
is zero, while over-titration produces a deflection in the opposite direction.

Application to the Determination of Boron in Plant Material†

The dried material is ashed in the presence of calcium oxide to prevent
loss of boron and the residue is extracted with hydrochloric acid.   Phosphate
and other interfering ions are removed by treatment with lead nitrate
solution and sodium bicarbonate, after which carbon dioxide is expelled
by acidification and boiling.   The solution is then adjusted potentiometrically
to a predetermined pH and titrated with standard sodium hydroxide as
described above.   Quantities of boron as small as 0·2 mg. may be determined
with a precision of $\pm 0·01$ mg.
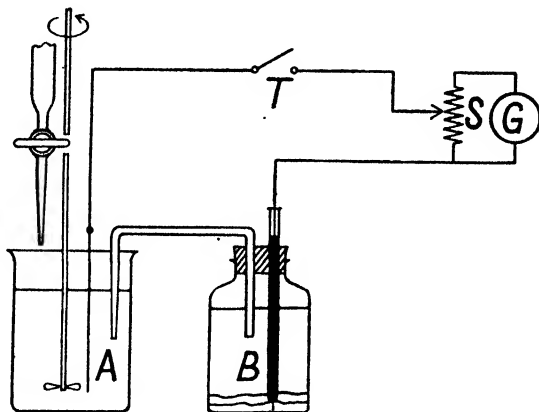
Fig. V.19.   Apparatus for Potentiometric Titration of Boron.
A, quinhydrone electrode; B, 0·7N calomel electrode; G, galvanometer; S, shunt;
T, tapping key.

APPARATUS. This is depicted in fig. V.19.   Use of boron-free glass is
not essential, provided heating in alkaline solution is not prolonged.   The
indicator electrode consists of a 3 in. length of stout platinum wire, while
the galvanometer has a maximum sensitivity of 0·025 microampere per
division, adjustable by means of shunt S.

REAGENTS.

1. *Calcium oxide.*

2. *Sodium bicarbonate.*

3. *Mannitol.* The blank titration for 5 g. must not exceed 0·1 ml. of
0·0231N sodium hydroxide solution.

    * Wilcox, *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 341.
    † Ruehle and Shock, ibid., 1945, **17**, 453.

4. 1N *lead nitrate.* Dissolve 165 g. in water and dilute to 1 l.

5. 2N *hydrochloric acid.*

6. 0·02N *hydrochloric acid.*

7. 0·5N *sodium hydroxide, free from carbonate.*

8. 0·0231N *sodium hydroxide, free from carbonate.* Standardise electrometrically against boric acid solution and store in paraffin-waxed bottles.

$$1 \text{ ml.} \equiv 0·25 \text{ mg. of boron.}$$

9. *Standard boric acid solution.* Dissolve 0·5716 g. of dry H_3BO_3 in water and dilute to 1 l.

$$1 \text{ ml.} \equiv 0·1 \text{ mg. of boron.}$$

METHOD. The material, freed from foreign matter without excessive washing, is dried at 70° C., ground, and again dried to constant weight. From 5–25 g. of the dried sample, containing not more than 2 mg. of boron, are weighed on to glazed paper and well mixed with calcium oxide (0·1 g. per gram of sample). The mixture is transferred to a platinum or porcelain dish and ignited as completely as possible in a muffle at low red heat.

When cold, the ash is moistened with water, and, after covering the basin with a watch-glass, is made strongly acid by adding 15–20 ml. of 6N hydrochloric acid. After heating for 30 minutes on a steam-bath, the solution is cooled and transferred to a beaker. Lead nitrate solution (1 ml. per gram of sample) is added, followed by 1 g. portions of sodium bicarbonate until a precipitate forms. The mixture is heated on a steam bath, a few drops of bromothymol blue are added, and more bicarbonate is introduced until the solution is blue-green in colour (pH $ca.$ 7).

After cooling, the mixture is transferred to a 250 ml. graduated flask and made up to the mark. It is then filtered through a dry paper, and an aliquot is acidified with 6N hydrochloric acid. This is heated to boiling, stirring cautiously, then vigorously, to expel carbon dioxide. Heating is discontinued, the solution is made alkaline with 0·5N sodium hydroxide, then acid with 2N hydrochloric acid, adding 5–10 drops of the latter in excess. Water is added to bring the volume to about 300 ml. and the solution is again raised to boiling for a few minutes (not longer) and stirred; the solution is then cooled.

Stirrer and electrodes having been introduced, 0·5N sodium hydroxide is added until the solution is approximately neutral; quinhydrone ($ca.$ 0·2 g.) is then added. On depressing the tapping key, the galvanometer deflection should be small; the sensitivity is then increased and the deflection is brought to zero by addition, as required, of 0·0231N sodium hydroxide or of 0·02N hydrochloric acid.

After reducing the galvanometer sensitivity, 5·0±0·1 g. of mannitol are added. The solution is then titrated with 0·0231N sodium hydroxide to

zero deflection, and the sensitivity of the galvanometer is increased to its original setting as the titration proceeds.

A blank correction, obtained by repeating the procedure with ashless filter-paper or other boron-free organic material, should be deducted from the volume of alkali required.

Example 2: The Determination of Microgram Quantities of Arsenic*

PRINCIPLES. On the micro scale trivalent arsenic may be conveniently titrated potentiometrically in the presence of hydrochloric acid with potassium bromate solution.† The method is suitable not only for small quantities of arsenic but also when the latter is present at high dilution and a fraction of a milligram of arsenic contained in 10 ml. of solution may be determined accurately. In small volumes of solution, amounts of arsenic of the order of a few micrograms may be titrated with an error of less than 1%.

The solution to be examined is strongly acidified with hydrochloric acid after adding a trace of potassium bromide. Titration with standard potassium bromate then follows, and at the end-point a bright platinum electrode inserted in the solution suffers a marked change in potential. However, since the interaction between the bromate and bromide ions is not instantaneous, the end-point must be approached slowly.

Even minute traces of organic matter interfere with the accuracy of this ultra-micro titration.

REAGENTS.
1. 5N *hydrochloric acid.*
2. 0·1000N *potassium bromate solution* (2·784 g. per litre; 1 ml. $\equiv$ 3·746 mg. of As^{III}). Dilute as required.
3. *Potassium bromide.*

METHOD. The drop-scale technique of Schwarz (p. 423), with a platinum ring electrode, is used. About 0·005 ml. of the solution, initially faintly alkaline and containing from 1–20 μg. of trivalent arsenic, is transferred to the ring electrode. A fragment of potassium bromide (*ca*. 1 mg.) and 0·003 ml. of 5N hydrochloric acid are then added. Depending upon the amount of arsenic present, titration is carried out either with 0·1N or with 0·01N potassium bromate. When approaching the end-point, some 30 seconds should elapse between addition of reagent and reading of the electrometer. Since a very marked change in potential occurs, the end-point can be determined without plotting the readings.

Even smaller quantities of arsenic than 1 μg. may be similarly determined if 0·005N or 0·001N potassium bromate be employed.

* Schwarz, *Mikrochem.*, 1933, 13, 16.
† Zintl and Betz, *Z. anal. Chem.*, 1928, 74, 330.

Example 3: The Determination of Water

PRINCIPLES. The end-point in the Karl Fischer titration (see p. 184) may be detected electrometrically with considerable precision. The latter, unlike that of the visual method, is of course unaffected when dark-coloured substances are examined. With this technique, addition of an excess of reagent followed by back titration with standard water solution is to be recommended.*

To detect the end-point potentiometrically, a platinum-tungsten electrode system (see p. 415) may be used.† The potential jump is, however, only about 20 millivolts. The "dead stop" method (see p. 415) is simpler and gives excellent results.‡ An E.M.F. of about 10 milli-volts is applied to two platinum electrodes immersed in Karl Fischer reagent, when a current of several micro-amperes flows. Addition of standard water solution has little effect upon the current until the end-point is reached, when the current falls abruptly almost to zero. The reaction is characterised by a lag of a few seconds, so that the titration should not be carried out too rapidly.

Application on the Micro Scale§

The following procedure, which was developed for the examination of penicillin sodium salt, is applicable to the determination of moisture in the wide variety of substances which do not react with the Karl Fischer reagent (see p. 185). With rigid exclusion of extraneous moisture, excess of the reagent is added to the sample. Back titration with aqueous methyl alcohol then follows. In this manner, quantities of from 1–25 mg. of water may be determined with a precision of from ±0·02–0·1 mg.

APPARATUS. The titration cell, which is designed to permit rigid exclusion of moisture, is shown in fig. V.20.

Fig. V.20.   Titration Cell for Use with Karl Fischer Reagent.

It is constructed from a 16 mm. diameter test-tube and is about 50 mm. long. Electrodes of 26 gauge platinum wire are sealed through the bottom of the vessel. When in use, the cell is closed by a sleeved rubber serum-bottle stopper. The micro-burettes containing respectively Karl Fischer reagent and standard water solution deliver through 22 gauge hypodermic syringe needles (see p. 152), which may be thrust through the stopper of the cell.

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* See, however, Acker and Frediani, *Ind. Eng. Chem.* (Anal. Edn.), 1945, **17**, 793; Carter and Williamson, *Analyst*, 1945, **70**, 369. These workers recommend direct titration for the determination of water in oils and in synthetic resin solutions respectively.

† Almy, Griffin, and Wilcox, *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 392.

‡ Wernimont and Hopkinson, ibid., 1943, **15**, 272; McKinney and Hall, ibid., 1943, **15**, 460; Levy, Murtaugh and Rosenblatt, ibid., 1945, **17**, 193.

§ Levy, Murtaugh, and Rosenblatt, ibid., 1945, **17**, 193.

To equalise the pressure, a 27 gauge needle is also inserted and serves as a vent. The air supply to the burettes must be thoroughly dry.

For detection of the end-point, the electrometric apparatus referred to on p. 427 may be conveniently employed.

REAGENTS.

1. *Karl Fischer reagent* (see p. 185). Standardise by titration with standard water solution in methyl or ethyl alcohol immediately before use.

2. *Aqueous methyl alcohol.* Add 2–5 g. of water to 1 l. of anhydrous methyl alcohol. Standardise by titrating portions of Karl Fischer reagent.

METHOD. The titration cell and rubber stopper are dried at 105° C. and stored in a phosphorus pentoxide desiccator until required for use. The sample, which should contain from 2–25 mg. of water, is weighed out and rapidly transferred to the cell, the stopper being fitted immediately. The venting needle is inserted, followed by that of the burette containing Karl Fischer reagent. The latter is run in until decolorisation ceases. Even with strongly coloured samples, approximate recognition of this point is usually possible. An excess of from 5–10% is then added and the needles are withdrawn. The cell is then shaken until the solids have dissolved. The venting needle is then replaced and the electrometric apparatus is connected to the electrodes. Aqueous methyl alcohol is added from the second micro-burette until decolorisation commences, the mixture being agitated by gently tapping the cell. Addition is then continued dropwise until the fluorescent pattern of the electron ray indicator shrinks to its minimum area.

TABLE I

FURTHER EXAMPLES OF POTENTIOMETRIC TECHNIQUES OF MICRO-ANALYSIS

Analysis	References
Determination of Metals	
Arsenic: drop-scale titration.	Zintl, E., and Betz, K., *Z. Anal. Chem.*, 1928, **74**, 330.
	Schwarz, K., *Mikrochem.*, 1933, **13**, 6.
Iron: drop-scale titration. titration with dichromate.	Schwarz, K., loc. cit. Benedetti-Pichler, A. A., *Z. Anal. Chem.*, 1928, **73**, 200.
Lead: drop-scale titration reaction.	Schwarz, K., *Mikrochem.*, 1933, **13**, 6.
Mercury: titration with iodide.	Schwarz, K., and Kantor, T., *Mikrochem.*, 1933, **13**, 225.
Silver: drop-scale titration with iodide.	Zürcher, M., and Hoepe, *Helv. Chim. Acta*, 1938, **21**, 1272.
titration with iodide.	Kolthoff, I. M., and Lingane, J. J., *J. Amer. Chem. Soc.*, 1936, **58**, 2457.
Thallium: after precipitation with thionalide.	Berg, R., and Fahrenkamp, E. S., *Mikrochim. Acta*, 1937, **1**, 64.
pH Determination (review).	Stock, J. T., and Fill, M. A., *Metallurgia*, 1946, **32**, 219, 271.
Determination of Acid Radicals	
Chloride.	Furman, N. H., and Low, G. W., *J. Amer. Chem. Soc.*, 1935, **57**, 1585.
	Schwarz, K., and Schlösser, C., *Mikrochem.*, 1933, **13**, 6, 18.
	Zintl, E., and Betz, K., *Z. Anal. Chem.*, 1928, **74**, 330.
	Linderstrøm-Lang, K., Palmer, A. H., and Holter, H., *Z. Physiol. Chem.*, 1935, **231**, 236.
Bromide: in presence of large amounts of chloride.	Vladimirov, G. E., and Epstein, J. A., *Mikrochem.*, 1935, **18**, 58.
	Zintl, E., and Betz, K., *Z. Anal. Chem.*, 1928, **74**, 330.
Selenite: titration with lead nitrate.	Ripan-Tilici, R., *Z. Anal. Chem.*, 1938, **114**, 412.
Organic Compounds	
Carbohydrates: titration with Fehling's solution.	Niederl, J. B., and Müller, R. H., *J. Amer. Chem. Soc.*, 1929, **51**, 1356.
Phenols: titration with bromide-bromate. titration with hypobromite.	Bielenberg, W., and Kühn, K., *Z. Elektrochem.*, 1943, **49**, 171; *Z. Anal. Chem.*, 1943, **126**, 88. Chirkov, S. K., *J. Applied Chem.*, *U.S.S.R.*, 1944, **17**, 31.
Acids: titration with alkali using glass electrode.	Catch, J. R., Cook, A. H., and Kitchener, J. A., *J. Chem. Soc.*, 1945, 319; Ingold, W., *Helv. Chim. Acta*, 1946, **29**, 1929.
Amino-acids: Formol titration using glass electrode.	Janke, A., and Mikschik, E., *Mikrochem.*, 1939, **27**, 176.

POLAROGRAPHY

Introduction

WHEN a solution of an electro-reducible or an electro-oxidisable substance is electrolysed using a succession of small mercury drops falling regularly from a fine capillary tube as one electrode, it is frequently possible to construct a reproducible current-voltage curve. From an examination of this, information both as to the identity and to the concentration of the substance present may be obtained.* In 1924, Heyrovsky and Shikata† invented the *polarograph*, by which the current-voltage curve is recorded automatically. This rendered the method much more useful, owing to simplification of manipulation and great saving of time.

The polarographic method has several characteristic features which make it particularly useful to the micro-analyst; thus:

(i) Very dilute solutions are used—the usual limits of concentration are from 10^{-5}–10^{-2} molar. Further, it is possible to work with small volumes. Cells of capacity as small as 2 ml. are available commercially, while an arrangement for examining volumes of about 0·005 ml. has been described by Majer.‡

(ii) Purely chemical operations are greatly simplified. It usually suffices to remove the bulk of an interfering substance. If this is done by precipitation, filtration can sometimes be avoided, the precipitate being allowed to settle as completely as possible and a portion of the supernatant solution withdrawn for examination.

(iii) Apart from the saving of time in preparing the solution, actual determination with a recording instrument is rapid, and a permanent record is available for reference.

(iv) Besides being applicable to ionised substances such as metallic salts, many non-ionised substances (e.g. dissolved oxygen, numerous organic compounds, etc.) may be determined. For this reason the method is often useful in biochemical studies.

(v) Since the total current passing during an average determination is very small, the test solution is for all practical purposes unchanged. Hence the determination may be repeated several times on the same solution, allowing a doubtful result to be checked.

* Heyrovsky, *Chem. Listy*, 1922, **16**, 256; *Phil. Mag.*, 1923, **45**, 303.
† *Rec. trav. chim.*, 1925, **44**, 496.
‡ *Mikrochem.*, 1935, **18**, 74.

GENERAL PRINCIPLES

1. The Electrode System. The apparatus is represented diagrammatically in fig. V.21. The *dropping mercury electrode* is here depicted as acting as the *cathode*. *Cathodic* processes, i.e. those involving *reduction*, are much more common in polarography than are *anodic*, or *oxidation*, processes. The simplest possible anode, viz. a pool of mercury at the bottom of the vessel, is shown. Whatever the nature of this second electrode, it is essential that its area should be sufficient to allow it to be regarded as incapable of becoming polarised. It serves as a *reference* or *quiet* electrode, permitting all effects observed to be attributed to polarisation and other phenomena occurring at the dropping electrode (sometimes termed the *indicator electrode*).

The potentiometer device P allows any E.M.F. up to about 3 volts to be applied to the cell, and to be gradually increased or decreased as desired. The current flowing, which rarely exceeds 50 microamperes, and is usually much less, is measured by galvanometer G. Owing to the growth and fall of the mercury drops, the current is pulsating. Hence a galvanometer of long period is used and devices to damp the galvanometer oscillations may be incorporated. The shunt S permits an appropriate galvanometer sensitivity to be selected, so that a wide range of substances and concentrations may be handled.
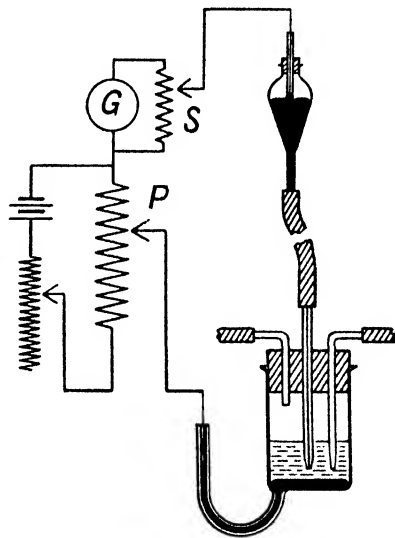
Fig. V.21. Basic Apparatus for Polarographic Analysis.

2. The Supporting Electrolyte. Electrolysis is nearly always carried out in a solution which contains, in addition to the substance to be determined, a considerably greater concentration of *supporting electrolyte* (also termed *indifferent electrolyte*). A "supporting electrolyte" is one which has a decomposition potential considerably higher than the substance being determined. Salts of the alkali metals, or of ammonium, are frequently employed. The purpose of the supporting electrolyte is to carry the current through the bulk of the solution, thus ensuring that the substance to be determined, if charged, does not *migrate* to the dropping mercury electrode (see Section 3). Interpretation of the results is thus facilitated.

3. Polarographic Waves. Suppose that an oxygen-free solution of pure potassium chloride is placed in the cell and a polarogram* recorded. The general shape will be that of Curve *I*, fig. V.22. Over the greater part of the polarogram only a very small current flows. This current, which is termed the *residual current*, increases linearly with the applied E.M.F. It is due partly to minute traces of impurities and partly to the charging of each new mercury drop. When the *decomposition-* or *reduction*-potential is reached, the current rises rapidly with further increase in applied E.M.F. until the galvanometer spot is deflected off the scale.

If a small concentration of an electro-reducible substance—a salt of zinc, for example—be added, and the solution again subjected to increasing applied E.M.F., a polarogram of Type *II*, fig. V.22, is obtained. At first
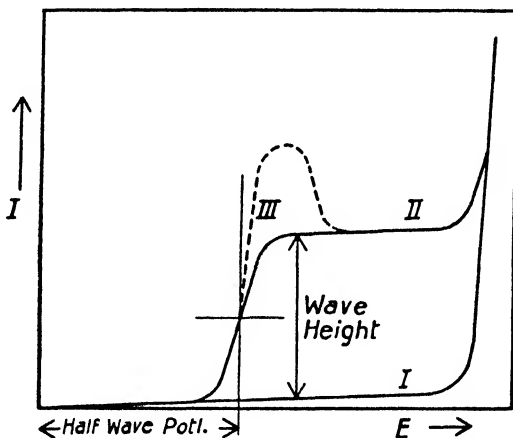
Fig. V.22. Typical Polarograms of Deoxygenated Solutions.
Curve *I*: Supporting electrolyte only.
Curve *II*: Supporting electrolyte containing substance to be determined.
Curve *III*: Distortion by maximum-formation.

only the residual current flows. Then, as the reduction potential of the reducible substance is reached, the current increases rapidly. The increase is not indefinite, however; the curve flattens out again, and the current shows no further marked change until the reduction potential of the indifferent electrolyte is reached. The S-shaped portion of the curve is termed a *polarographic step* or *wave*, and the current flowing in the region indicated by the flat portion of the curve succeeding the wave is termed the *diffusion current*. After subtracting the appropriate residual current, it is frequently found that the diffusion current—or, as it is often expressed, the

* The term "polarogram" is used throughout to mean a current-voltage curve obtained by use of the dropping mercury electrode, irrespective of whether the result is recorded automatically or plotted on graph paper from a series of individual readings.

wave height—is directly proportional to the concentration of reducible substance in solution. Quantitative polarography is based on this simple relation.

4. The Wave Height. The fact which is of extreme importance to the analyst, that the height of a polarographic wave is related so simply to the concentration of the reducible substance, is explained as follows.

When the decomposition potential of the reducible substance has been exceeded, electrolysis commences—the current at first rises rapidly as the applied E.M.F. is further increased. As electrolysis proceeds, however, the layer of solution immediately adjacent to the dropping electrode rapidly becomes depleted of the reducible substance and a state of *concentration polarisation* sets in. Further amounts of reducible substance can reach the depleted cathode layer in two ways:

(i) By *diffusion* from the bulk of the solution.

(ii) If the substance is charged, i.e. is an ion, by *migration* from the bulk of the solution.

The presence of a large excess of supporting electrolyte, the ions of which carry practically the whole of the current through the solution, reduces the migration effect to negligible proportions. Accordingly, the rate of electrolysis and hence the current flowing is governed by the rate of diffusion of the reducible substance from the bulk of the solution into the cathode layer. The rate of diffusion is independent of the applied E.M.F., hence the current remains practically constant until some other electrode reaction, such as the reduction of the indifferent electrolyte, occurs. The rate of diffusion, other conditions being kept constant, depends directly upon the concentration of the substance being determined. Accordingly, the diffusion current is a direct measure of this concentration.

5. The Half-wave Potential. The polarographic waves of various substances occur at different positions on the potential scale, hence this may serve as a means of identification. The earlier workers recorded the reduction potentials of the substances they examined. However, the reduction potential of a given substance is not constant, but varies both with the concentration and with the particular dropping electrode in use. It was shown by Heyrovsky and Ilkovic* that the potential at a point on the wave at which the current has obtained one-half its limiting or diffusion current value is far more characteristic, being independent of the concentration of the substance and of the properties of the dropping electrode. The potential at this point is termed the *half-wave potential* of the substance. It is usual to report half-wave potentials using the saturated calomel electrode (S.C.E.) as a standard of reference.

6. The Ilkovic Equation. Besides the concentration of the reducible substance, there are several other factors which also influence the magnitude

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* *Collection Czechoslovak Chem. Commun.*, 1935, **7**, 198.

of the diffusion current. These factors, together with the concentration, are related to the diffusion current by means of a single equation first derived by Ilkovic* in 1934:

$$I_d = 605 . n . D^{1/2} . C . m^{2/3} . t^{1/6},$$

where I_d is the *average* diffusion current in microamperes (as previously pointed out, the current is pulsating), n the number of faradays of electricity involved per mole of the reducible substance, D the diffusion coefficient of the substance, C its concentration (millimoles per litre), m the flow of mercury from the dropping electrode (milligram per second), and t the drop-time in seconds.†

The diffusion coefficient D of the reducible substance depends on several factors, such as the total salt concentration and the viscosity of the solution. The latter factor has a considerable temperature coefficient, hence so have both D and I_d. Accordingly, the use of a thermostat is recommended. Control of temperature to within $\pm 0.5°$ is quite sufficient.

The factors m and t are characteristic of the particular dropping electrode in use. For a given capillary, m depends on the pressure of mercury and is practically independent of the medium in which the drops form. Since the temperature coefficient of m is much smaller than that of D, m can be regarded as constant when using a fixed pressure of mercury, as is usual in practical polarography. The drop-time t depends not only on the mercury pressure, but also on the interfacial tension at the mercury-solution interface. Hence t varies with the nature of the solution. Since the interfacial tension also depends on the potential, t varies continuously as the polarogram is being traced, rising to a maximum, then decreasing as the applied E.M.F. is further increased (fig. V.23). Fortunately, the Ilkovic equation involves t as the one-sixth power only, hence its variations are not so serious as might be expected. The effect needs to be borne in mind, however, when analysing mixtures of substances whose reduction potentials are widely separated.

Fig. V.23.   Variation of Drop-time with Potential.

* *Collection Czechoslovak Chem. Commun.*, 1934, **6**, 498; *J. chim. phys.*, 1938, **35**, 129.
    † "Polarography" (Interscience Publishers, Inc., New York, 1941), p. 39. But cf. Gaskin and Whalley, *Chemistry and Industry*, 1943, **62**, 442.

The product $m^{2/3}t^{1/6}$ is important, as it permits correlation of results obtained using different dropping electrodes, and the inclusion of its value when reporting polarographic investigations is recommended.

7. Effect of Dissolved Oxygen. Certain substances, e.g. oxygen, give rise to multiple waves, due to stepwise reduction. Fig. V.24 shows the waves yielded by $0 \cdot 1 M$ potassium chloride solution made from ordinary, air-containing distilled water (a trace of methyl red solution was added to remove irregularities termed *maxima*, as discussed in Section 9). At ordinary temperatures, oxygen dissolves in water to the extent of about 8 mg. (i.e. $2 \cdot 5 \times 10^{-4}$ mole) per litre. Accordingly, most test solutions contain sufficient oxygen to produce waves which may be actually greater in height than those of the substance to be determined. Unless the latter is present in sufficient concentration to form a large wave, compared with which the oxygen waves are negligible,* dissolved oxygen usually has to be removed. This is usually done by sweeping out with a stream of hydrogen or a similar gas (see p. 450). In neutral or alkaline solution, oxygen may be destroyed by addition of a little sodium sulphite to the test solution.† An example of this procedure is given on p. 468.
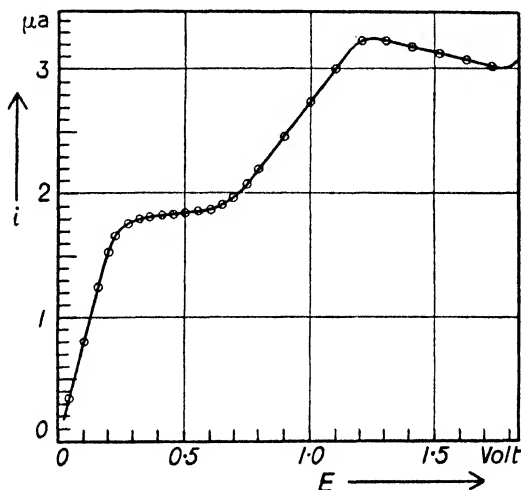
Fig. V.24. Polarographic Waves of Dissolved Oxygen.

8. Analysis of Mixtures. When several reducible substances are present in approximately similar concentrations, a polarogram consisting of a series of waves is obtained. Provided that the substances do not mutually interfere and that their waves are sufficiently separated, it is possible to estimate the concentration of each substance from the height of its particular wave. An example of this is given on p. 467.

9. Maxima. It is sometimes found that the wave crests are distorted by peculiar humps or peaks termed *maxima*, as shown diagrammatically in Curve *III*, fig. V.22. These irregularities are a nuisance in quantitative polarography, preventing accurate measurement of the wave-height. Maxima are attributed to adsorption phenomena, to stirring effects due to

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* See, for example, Haslam and Cross, *J. Soc. Chem. Ind.*, 1945, **64**, 259.
† Kolthoff and Laitinen, *Science*, 1940, **92**, 152.

uneven potential distribution at the surface of the dropping mercury electrode, or to both causes. Fortunately, the addition to the test solution of traces of certain dyestuffs, colloids, etc., often suppresses completely an undesirable maximum, so that a wave of normal shape is obtained. Methyl red and gelatin are examples of *maximum suppressors*; however, the most suitable agent for a particular determination can only be found by trial. Maximum suppressors should be used with caution, as not only may the maxima be removed, but also considerable alteration in the shape, position, and magnitude of the waves may occur.*

* Kolthoff and Barnum, *J. Amer. Chem. Soc.*, 1941, **63**, 520; Stock, *J. Chem. Soc.*, 1944, 427.

POLAROGRAPHIC APPARATUS

1. Preparation of the Dropping Electrode. The fine capillary from which the mercury drops issue may be made by drawing out Pyrex tubing of about 0·5 mm. bore and 5–6 mm. outside diameter. The walls should be allowed to thicken up so that the drawn-out portion is not too fragile.

More robust capillaries can be made from short lengths of hard glass clinical thermometer tubing of bore 0·03–0·05 mm. diameter. To facilitate connection to the mercury supply these short tips are sealed by the following method to 10–15 cm. lengths of wide-bore tubing which has a slightly greater outside diameter.

One end of a piece of thermometer tubing is sealed, and a pressure of about 10 lb. per square inch, obtained from a cylinder of oxygen or other non-inflammable gas, is applied to the open end. The sealed end is then heated in the blowpipe flame, when a bubble appears and bursts, or may be chipped open with a glass-knife.* The expanded end is cautiously sealed on to the wider tube, using a small flame directed well away from the fine part of the tip. To eliminate failures it is best to apply a rapid sorting test before commencing adjustment. The upper end is connected to a water pump, while the tip is immersed in pure mercury. If the capillary is unobstructed, a bead of mercury will have formed above the seal within a minute or so.

Each capillary should be tested and adjusted to correct dropping time in potassium chloride solution of about $0·1M$ strength. For capillaries made by drawing out, this is done as follows:

The capillary is attached by a glass-to-glass joint inside heavy rubber tubing to the drawn-down lower end of a glass tube about 70 cm. long and 1 cm. bore, which is held vertically in a clamp. Pure, dry mercury is poured into the tube until the height of the column, measured from the tip of the capillary to the meniscus, is about 60 cm. The tip of the capillary is then immersed in potassium chloride solution and cut down until the drop-time lies between 3 and 5 seconds, and preferably towards the lower limit.

The drop-time is best determined by timing the formation of 10 or more successive drops and taking the average. A few millimetres only should be removed from the tip at each adjustment, by making a fine scratch at the desired point (a sharp flake of carborundum crystal is useful for this purpose) and breaking off the excess. When adjusted, the capillary is rinsed, the mercury allowed to drop into distilled water for a few minutes, then all traces of moisture removed by strips of filter-paper while mercury is still flowing. The mercury is then poured and shaken from the reservoir tube, when the capillary may be detached without spillage. Water or solutions must not enter the capillary, or erratic dropping or even blockage will result.

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* See also Kahan, *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 549, for an alternative method of blowing out.

Within limits, the drop-time is inversely proportional to the pressure head, hence an over-adjusted capillary may drop at a satisfactory rate with a smaller head of mercury. If the head is made too small, the mercury flow stops abruptly. The working head should always be at least 5 cm. greater than the minimum for steady dropping, to ensure that the issue will not cease while a determination is being made.

Similar remarks apply to the adjustment of capillaries with sealed-on tips. However, it is not easy to break off short lengths of the stout tip. Hence after ascertaining the approximate length required by a preliminary trial, it is wise to fabricate the tip to be about 1 cm. or so longer than this, to determine the drop-time, and to cut off the calculated excess, taking advantage of the fact that, for a uniform capillary, the drop-time is proportional to the length. Finally the drop-time is redetermined; as before, this can be altered within limits by varying the pressure of mercury. Satisfactory capillaries should be stored separately in corked test-tubes, labelled with the drop-time and the approximate head of mercury with which this is obtained.

2. Assembly and Maintenance of the Dropping Electrode. The simplest method of supplying the necessary pressure of mercury to the capillary is by means of a reservoir arranged at the requisite height above the capillary tip and connected to it by a length of heavy-walled rubber tubing. Electrical connection is by means of a platinum wire dipping below the surface of the mercury in the reservoir. Before use, the rubber tubing is boiled out with caustic soda solution to remove sulphur then well washed and thoroughly dried.* After raising the capillary to permit removal from a solution, resetting to an identical pressure-head may not be easy. In the "Cambridge" dropping electrode assembly† this difficulty is overcome in an ingenious manner. The capillary and reservoir are attached to a carriage so that on lifting the capillary clear of the cell, the reservoir rises with it, thus maintaining a constant pressure of mercury.

If treated with reasonable care, a correctly fabricated capillary may be expected to have a very long life. Choking, caused by the use of impure mercury or by the entry of foreign matter at the tip, is the usual trouble encountered. The passage of heavy currents, due to the use of too high a concentration of the substance being determined, is deleterious; this applies specially when a metallic ion is being deposited, as the resulting amalgam may foul the capillary.

Only the purest mercury should be used. To remove base metals, aeration is preferable to treatment with nitric acid as in the Meyer method.‡ Wet

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* Various all-glass assemblies have also been recommended, mainly on the grounds of contamination of mercury by rubber. Though batches of tubing do differ, the author has never experienced trouble after proper treatment (cf. "Polarography," p. 243). Neoprene or similar synthetic material is satisfactory.

† Cambridge Instrument Co., Ltd., List No. 109.

‡ Wichers, *Rev. Sci. Instruments*, 1942, **13**, 502.

mercury should be dried by means of strips of blotting-paper, and then strained in the usual way through a pierced filter-paper. A simple device for aerating is shown in fig. V.25. The mercury is introduced until the wider part of the vessel is about half-full, and the suction tube is attached to a water pump adjusted so as to draw a slow stream of air bubbles through the mercury. This is continued for several days. Impurities are oxidised and rise as scum in the wider portion of the vessel. Clean mercury can then be poured from the inlet tube by tilting. Noble metals are, of course, not removed in this way. A small amount of platinum dissolved from the various contacts is the most likely contaminant of this nature. Accordingly, occasional distillation should be carried out.

To avoid blockages, the tip of the capillary should be immersed in dilute nitric acid for a few minutes before arresting the flow of mercury, and then well rinsed with distilled water. With the tip immersed in a beaker of distilled water, the pressure of mercury is steadily reduced until the flow of mercury just ceases. Alternatively, the tip may be dried carefully with strips of filter-paper and the pressure then decreased to stop the flow. A choked capillary may sometimes be cleared by expelling the mercury and then sucking through aqua regia by means of a water pump. Kahan* recommends iodine in potassium iodide solution as an effective cleansing agent. Before re-use the interior of

Fig. V.25. Apparatus for Purification of Mercury.

the capillary must be dried completely, since traces of water inevitably cause erratic behaviour. Drying is best accomplished by drawing a current of warm air, filtered through a plug of cotton-wool, through the capillary.

3. Polarographic Cells. Besides serving to contain the solution to be examined, the cell or electrolysis vessel must permit the ready insertion of the dropping electrode. Provision must also be made for a mercury pool or alternatively an external electrode, and also for passing a stream of gas through the solution for the purpose of removing dissolved oxygen. Further, the cell should be convenient for mounting in a thermostat when desired. In certain special cases, openings for the connection of a reference electrode and for the entry of the tip of a burette must also be provided.

Many different cells, two of which are shown in fig. V.26, have been designed. The original Heyrovsky pattern (a) is useful for fairly large volumes of solution, but is somewhat awkward to use in the thermostat.

Kolthoff and his co-workers* favour cells constructed from small beakers. A typical example is shown at (b). A very useful cell incorporating an external reference electrode has been described by Lingane and Laitinen.†

For ease of manipulation, the range of interchangeable cells designed by Jessop‡ is probably unsurpassed. With their complete absence of rubber stoppers, these cells can be rapidly mounted and dismounted with little fear of damaging the dropping electrode.

Cells of 2 and 20 ml. capacity are shown at (a) and (b) respectively in fig. V.27. A mercury pool is normally used, connection to it being made by a sealed-in platinum wire. The side-tube serves as an inlet for the gas stream. A bell-shaped cover attached to the dropping electrode dips into a little water placed in the annular portion surrounding the upper part of
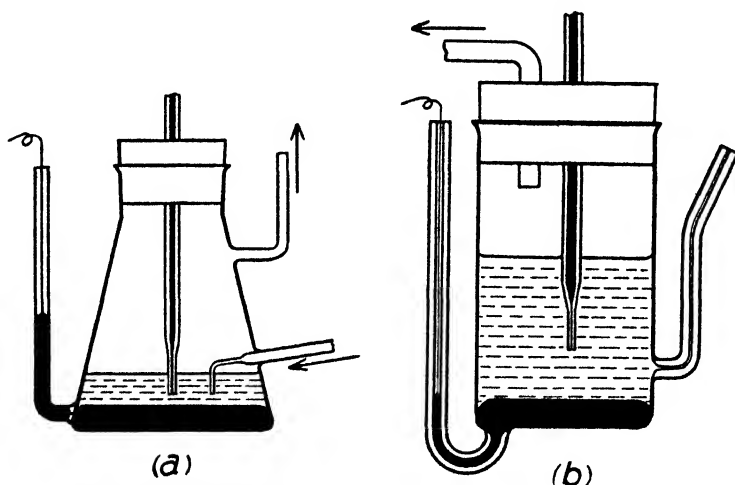
Fig. V.26.   Typical Polarographic Cells.
(a) Heyrovsky pattern.   (b) Beaker pattern.

the cell. This permits ready escape of gas, but prevents entry of air. The cell can be modified to permit the use of an external electrode. This is constructed to slip into a second side-tube as shown at C. A compact thermostat to hold four of these small cells is available.

4. The Electrical System. The polarograph is simple to use, provides a permanent record of every analysis, and saves a great deal of time. Hence it is invaluable to routine work. However, the simpler manual apparatus, though much more tedious to use, is of great value for occasional investigations not warranting the acquisition of a special, expensive instrument.

* J. Amer. Chem. Soc., 1939, **61**, 825.
† Ind. Eng. Chem. (Anal. Edn.), 1939, **11**, 504.
‡ Cambridge Instrument Co., Ltd., List No. 109.

(*A*) *The Polarising Unit.* This is a device for applying to the electrodes a known E.M.F. ranging from zero to about 3 volts. The simplest arrangement, in which the applied E.M.F. is measured directly by a calibrated voltmeter,
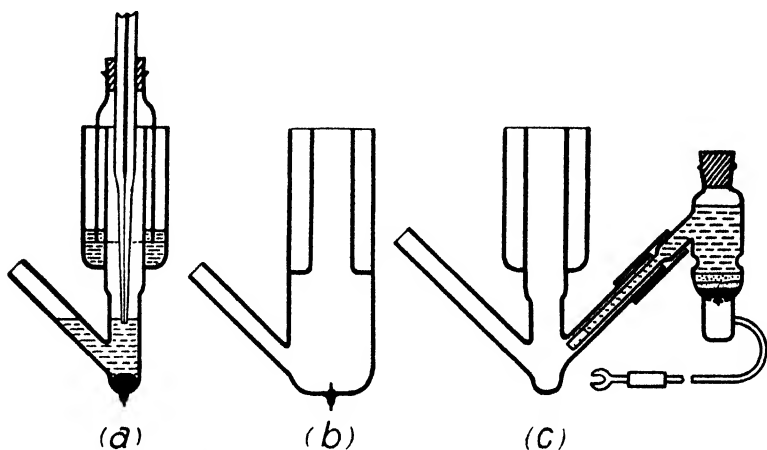
Fig. V.27. "Cambridge" Polarographic Cells.

(*a*) 2 ml. cell. (*b*) 20 ml. cell. (*c*) Modification for use with external reference electrode.

is depicted schematically in fig. V.28. Power is supplied by a 4 volt accumulator *B*. Two wire-wound radio-type potentiometers, R_1 and R_2,
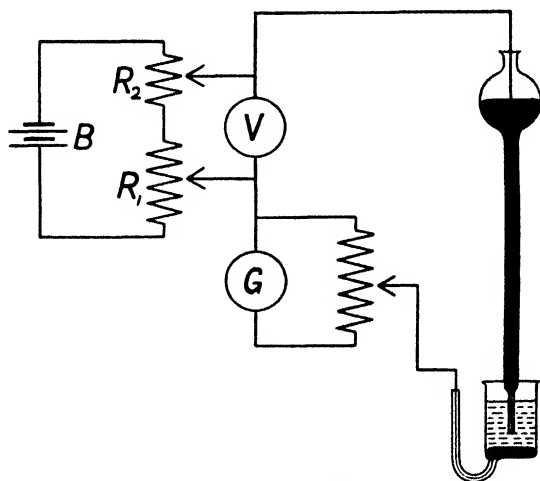
Fig. V.28. Polarising Unit for Manual Apparatus.

of 1,000 and 10 ohms resistance respectively, serve as coarse and fine adjustments of the applied E.M.F. The scale of the voltmeter *V* should preferably be divided into tenths of a volt, allowing hundredths to be estimated.

An alternative arrangement is shown in fig. V.29. The regulating resistance *R* permits the *total* potential drop along the metre slide wire *XY* to be so adjusted that each millimetre of the wire corresponds to a convenient number of millivolts. Any desired voltage up to the maximum may then be obtained by adjustment of slider *S*. If the potential drop along *XY* is kept constant, then the voltmeter *V* needs to be calibrated only at this point on its scale. Hence a comparatively cheap instrument can be useful.*

The necessary applied E.M.F. may also be obtained from a dial potentiometer of the usual type. The desired value is set up on the dials and slide wire and taken from the terminals normally used for the input.

(*B*) *Measurement of Current.* As already pointed out (p. 435), the current to be measured is small and pulsating. A mirror galvanometer with a long period—20 seconds or greater—is usually recommended as a suitable measuring
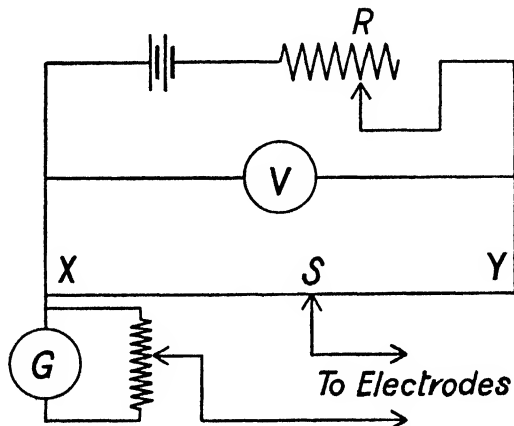
Fig. V.29. Alternative Arrangements of Manual Apparatus.

device. It should have a maximum sensitivity of 0·1 or, better, 0·01 microampere per millimetre, and should be provided with a calibrated universal shunt in order that the sensitivity can be adjusted as required. In practice, instruments with a much shorter period may be used. The oscillations may be somewhat pronounced, but are usually perfectly reproducible. Muller* recommends the use of a slip of white paper to assist reading. This is moved slowly inwards from the extreme portion of the scale until the inner edge is just illuminated by the moving spot of light. The reading is then easily recorded. If desired, the minimum reading may be obtained by approaching from the opposite direction. By the use of special damping devices (*vide infra*), the oscillations may be greatly reduced. Galvanometers with a built-in lamp and scale are particularly convenient, as there is no fear of displacement of the various portions of the optical system.

* Muller, *J. Chem. Ed.*, 1941, **18**, 111.

Quite frequently, the diffusion current, or "wave height," is expressed in terms of millimetres deflection instead of in electrical units. For general analytical work this is quite satisfactory. However, it is often desirable to obtain the result in microamperes. This may be done by calibrating the galvanometer. A standard resistance (i.e. a decade or other resistance box) of about 10,000 ohms is arranged in series with the galvanometer and a small current passed through both. A convenient way of doing this is to use the dropping electrode system with a solution containing a suitable amount of readily reducible material, e.g. a salt of zinc, and to stop the increase of applied E.M.F. when the diffusion current region is reached. The voltage drop across the standard resistance is measured by means of a potentiometer and hence the current is readily calculated from Ohm's Law. The mean galvanometer deflection is noted. By adding to the cell small amounts of more concentrated zinc solution from a burette, the current is increased, and similar readings are taken after each increment. By this means, a current-deflection curve, which should be linear, may be obtained. It is convenient that each scale division should represent some simple fraction of a microampere, e.g. 0·1 or 0·01. While the calibration is in progress, the accuracy of the shunt should be checked by comparing galvanometer readings at different settings.

(C) *Galvanometer Damping Devices.* In certain cases it is desirable to suppress the diffusion current due to a large wave in order that a second, smaller wave may be examined at a suitable galvanometer sensitivity.

This is usually accomplished by passing a *counter-* or *compensating current* through the galvanometer so that the current due to the large wave is balanced out. Unfortunately, though the unwanted wave may be eliminated, the current pulsations associated with it remain, and, when the sensitivity is increased, cause unduly large galvanometer oscillations. These may be greatly reduced by connecting a condenser of large capacity across the shunt input. A good quality electrolytic condenser of 2,000 mfd. capacity is suitable. The net parallel resistance of the galvanometer and shunts should not be too low, or the damping effect will be insufficient. A value of about 2,000 ohms is satisfactory. This resistance varies with the setting of the shunt*: accordingly the degree of damping depends on the sensitivity selected, being least at maximum and minimum settings.

This method of damping is also very useful when using galvanometers of short period.† In fact, a microammeter of the pointer type may be employed in place of a mirror galvanometer,‡ rendering the apparatus more compact.

5. Recording Instruments. Instruments for recording automatically the current-voltage curve are of two types, viz. those employing th

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* Lingane and Kerlinger, *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 750
† Fill and Stock, *Trans. Faraday Soc.*, 1944, **40**, 502; Stock, *Analyst*, 1946, **71**, 585.
‡ Neuberger, *Z. anal. Chem.*, 1939, **116**, 1.

photographic method,* as was used in Heyrovsky and Shikata's original design,† and those in which the record is drawn by an electrically operated pen.‡

Fig. V.30 illustrates schematically the essentials of the photographic type of instrument. A sheet of photographic paper is clipped on to cylinder A, which can be rotated about its axis by electric motor C operating through
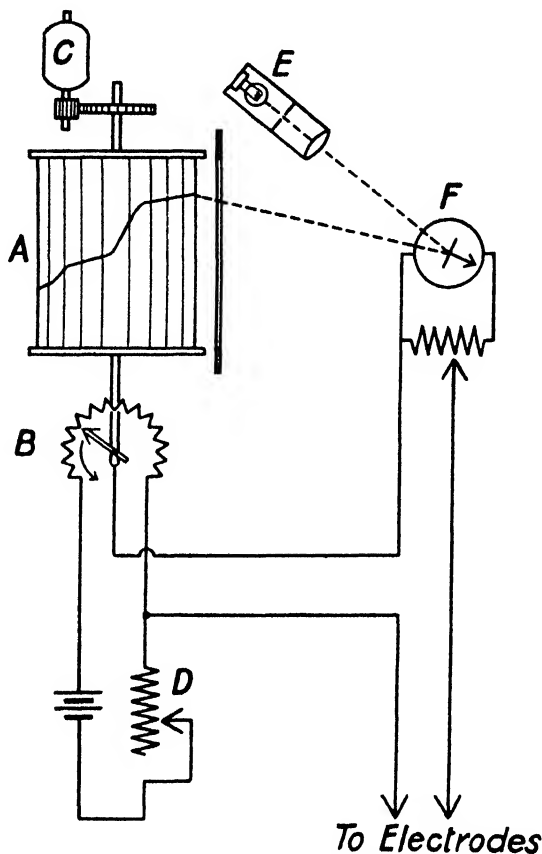
Fig. V.30. Schematic Arrangement for Photographic Recording.

suitable reduction gearing. Coupled to A and rotating with it is potentiometer drum B. By means of regulating resistance D the fall of potential across B is adjusted to a predetermined value. A narrow beam of light from lamp E is reflected from the mirror of galvanometer F and falls on a

* See, for example, Cambridge Instrument Co., Ltd., List 109; E. H. Sargent and Co., "Polarographic Analysis," Chicago, 1941.

† *Rec. trav. chim.*, 1925, **44**, 496.

‡ See, for example, Tinsley (Industrial Instruments), Ltd., Leaflet "Ink Recording Polarograph," London; Leeds and Northrup, Bulletin E-94 (1), Philadelphia, 1942.

slit arranged in front of the sensitive paper and parallel to the axis of A. The current passing through the electrolysis system is indicated by the position at which the spot of light passes through the slit. This is recorded by the paper. When the motor is started, the E.M.F. applied to the electrodes is steadily increased, and at the same time the paper begins to move past the slit. The polarogram is accordingly traced out by the combined motions of the paper and of the spot of light. To assist location along the voltage axis, an interrupter operated by the potentiometer drum causes an auxiliary lamp to illuminate momentarily the entire length of the slit, causing thin lines to be recorded at predetermined voltage intervals, usually every 0·1 volt. These lines locate the waves on the completed polarograms, and, incidentally, correct for any shrinkage of the paper during development,
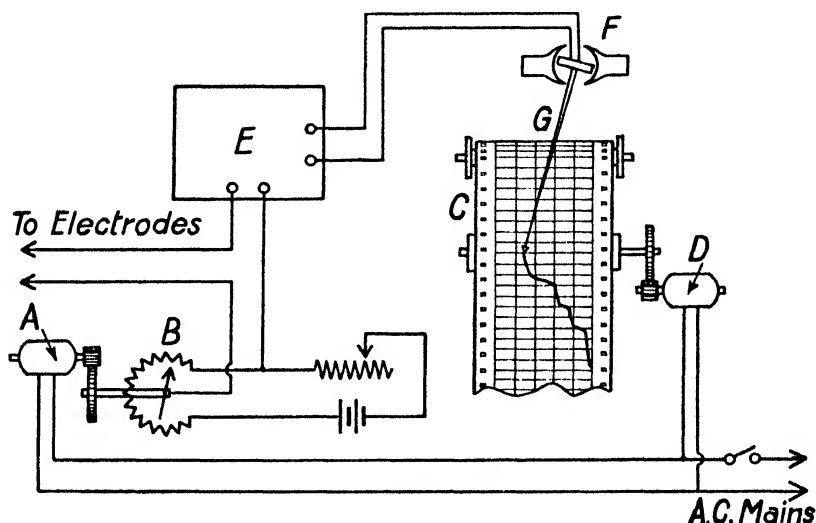
Fig. V.31.   Apparatus for the Ink Recording of Polarograms.

etc. Provision is made for the recording of several polarograms on a single sheet should this be desired.

Since the currents involved are so small, instruments of the pen type include an amplifying device the output of which is sufficient to operate the pen. The schematic arrangement of the apparatus is shown in Fig. V.31. Potentiometer drum B is driven through suitable reduction gearing by synchronous motor A, so that the E.M.F. applied to the electrodes is steadily increased. Strip-chart C is driven by a similar motor D, the gearing being so adjusted that the paper, which is ruled transversely in fractions of a volt and longitudinally in microamperes, moves past the pen at the same rate as the E.M.F. applied to the electrodes is increased. The current flowing through the electrolysis system is amplified by thermionic valve unit E

and is then sufficiently great to operate the movement F of the ink-recorder, which causes pen G to move across the width of the chart and to take up a position dependent upon the current flowing through the electrolysis system. The polarogram is thus traced out by the combined motions of the paper and the pen.

EXPERIMENTAL TECHNIQUES

1. General Considerations. Unless the drops of mercury issuing from the capillary grow and fall without interference, erratic results will be obtained. Accordingly, freedom from vibration should be looked for when selecting a position for fitting up a polarograph. A connection to the service mains is needed to feed the galvanometer lamps, etc.—care should be taken that the voltage and type of supply are suitable. Accumulators kept well charged and in good condition are needed for energising the polarising apparatus. To avoid voltage fluctuations, they should be connected directly and not used to feed a supply system common to other apparatus. Current obtained by rectifying A.C. from the mains is unsuitable. Particularly when using the manual form of the apparatus, it is desirable to work in a partially darkened room, using electric lighting when required. This facilitates observation of the light-spot on the galvanometer scale.

2. Preliminary Operations. Mercury is introduced into the dropping electrode assembly and the pressure is increased by raising the reservoir until mercury begins to issue from the capillary and to fall in large drops. The tip of the capillary is then immersed in distilled water and the mercury is allowed to drop for about 5 minutes. The distilled water is then replaced by an approximately normal solution of potassium chloride, and, by varying the pressure of mercury, the drop-time is adjusted to the desired rate. In the case of the "Cambridge" Polarograph this is $2\frac{1}{2}$–3 seconds per drop. Before arresting the flow, the pressure of mercury is noted and the tip of the capillary is well rinsed and immersed in distilled water.

3. The Test Solution. A solution which is one-tenth normal with respect to potassium chloride and which contains 100 mg. of cadmium per litre is a useful reference standard for testing and adjusting. It may be prepared by dissolving exactly 1 g. of pure cadmium in nitric acid and evaporating almost to dryness, taking the usual precautions to avoid loss by spray, etc. The residue is dissolved in distilled water and made up to 1 l. 10 ml. of this solution and an equal volume of normal potassium chloride solution are diluted to 100 ml. with distilled water to give a suitable mixture for each test.

A convenient volume of the prepared solution is introduced into the polarographic cell, so that the tip of the dropping electrode is about 5 mm. below the surface of the liquid and that the drops fall freely under the correct pressure of mercury. Dissolved oxygen is removed by passing a stream of

bubbles of nitrogen or hydrogen through the solution. Gas as supplied in cylinders is usually sufficiently pure for general work, but, when necessary, traces of oxygen may be removed by passing the gas through alkaline pyrogallol. The time required to de-oxygenate the solution varies from approximately 2 minutes with a micro-cell of 1 ml. capacity* to 20 minutes with a cell containing 20 ml. of liquid. In this respect, cells of small capacity are advantageous. Before proceeding with the electrolysis, the gas stream must be stopped; otherwise erratic results will be obtained.

4. Manipulation of Manual Apparatus. The electrical connections are completed, taking care that the dropping electrode is made the *cathode* of the electrolysis system. As a precaution, the galvanometer shunt should be switched to the position of lowest sensitivity and the test solution first examined as follows. The applied E.M.F. is first raised to 0·9–1·0 volt in order that the diffusion current of the cadmium ions may be obtained. The sensitivity of the galvanometer is then increased until the mean deflection is half or two-thirds of the maximum of the scale. The applied E.M.F. is returned to zero, and is then increased in steps of 0·05–0·1 volt, noting the exact E.M.F. and galvanometer deflection after each increment. When the reduction potential of cadmium is reached, the current begins to rise rapidly, and it is best to take readings at intervals of 10–25 millivolts. When the region of the diffusion current is reached the intervals may be increased once more. This procedure allows the shape of the polarogram to be readily defined by plotting on squared paper the current flowing against the corresponding value of applied E.M.F. The polarogram of an unknown solution can then be plotted in exactly the same way. Very often, when the location of a "wave" is known, as in routine analysis, it is unnecessary to plot the whole curve. A few readings are taken at voltages below that of the onset of the wave in order to define the slope of the (linear) residual current curve (see p. 436). A single reading is then taken at an applied E.M.F. at which the diffusion current flows. The residual current at this E.M.F. is obtained by extrapolation and subtracted from the observed diffusion current, as shown in fig. V.35. The value of the corrected diffusion current (wave height) enables the concentration of the wave-forming substance to be obtained from a calibration curve or by other suitable means.

Manipulation of the "Cambridge" Polarograph

1. *Preliminary Adjustments.* The switches "Lamps," "Projector Lamp," and "Ground Lamps" (fig. V.32) are moved to the downward position. By rotating knob P the potentiometer dial (seen on the extreme left on looking into O) is returned to zero. As each voltage-marking is passed, the white light affording general illumination of the scale should be interrupted. This is the device which produces the squared background of the polarogram.

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* Stock, *Analyst*, 1946, **71**, 583.

The battery switch is now moved to the "on" position and the knob L is rotated until the meter reads "Test." The sensitivity (knob M) is adjusted to 1/20 and knobs J and K are rotated to zero. The spot of light should be on the scale; if not, adjustment of knob N will cause it to appear, and will enable it to be brought to a reading of about 5 divisions. The instrument is now ready for taking records; to avoid fogging the photographic paper, the lamps are turned off before commencing adjustments to the camera.
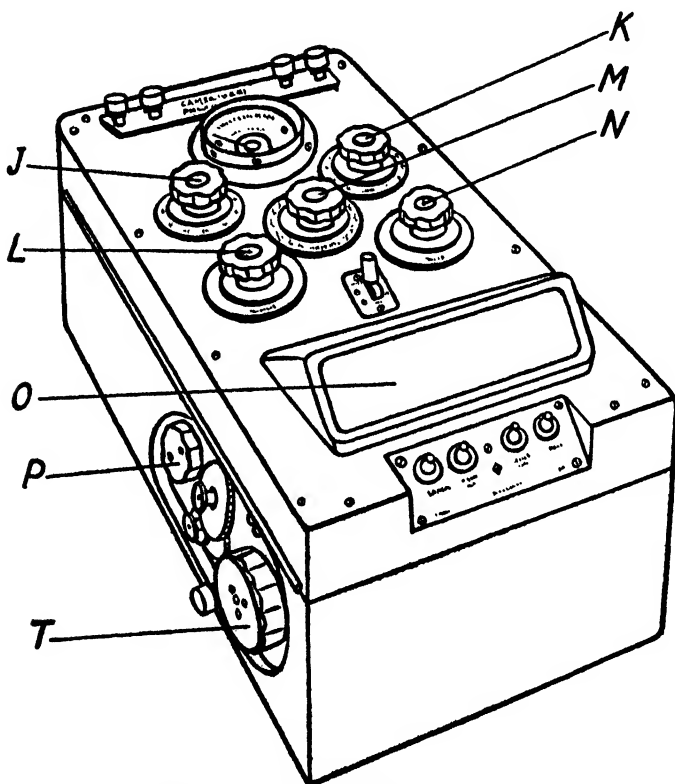
Fig. V.32. The "Cambridge" Polarograph.

2. *Loading the Camera.* Records are taken on $6\frac{3}{4}$ in. $\times 3\frac{1}{4}$ in. vigorous matt "gaslight" paper (Kodak "Velox" or Selo "Special Base 437 CJ" is suitable). This may be loaded into the paper-holder (fig. V.33) in shaded artificial light. On pushing drum catch A away from the handle, the drum can be easily removed from the cover. The drum is then held in the left hand and the spring clips are released by pushing knob B. With the sensitised side outwards, one end of the paper is inserted into clip C to rest against stop E. Having adjusted the paper squarely to bring the bottom edge of the paper against flange D, the clip is depressed. The paper is

then wrapped round the drum and the free end is inserted into the second clip F, which is depressed after drawing the paper tightly round the drum with one edge fitting against the flange. After inserting the drum into the cover, rotation will cause pin G to engage with the slot H in the cover. The cover is then locked in place by pushing the drum catch towards the handle. The paper-holder may be inserted into the camera in full daylight. To do this, knob T (fig. V.32) is pulled outwards until the pin disengages from the slotted disc and rotating until the camera dial (observed next to the potentiometer dial on looking into O) reads zero. After engaging the projection on the cover with the slot in the case, the paper-holder is pushed home into the camera, when a groove on the inner end of the drum should engage on a pin on the spindle of the camera dial. On lifting the drum catch and
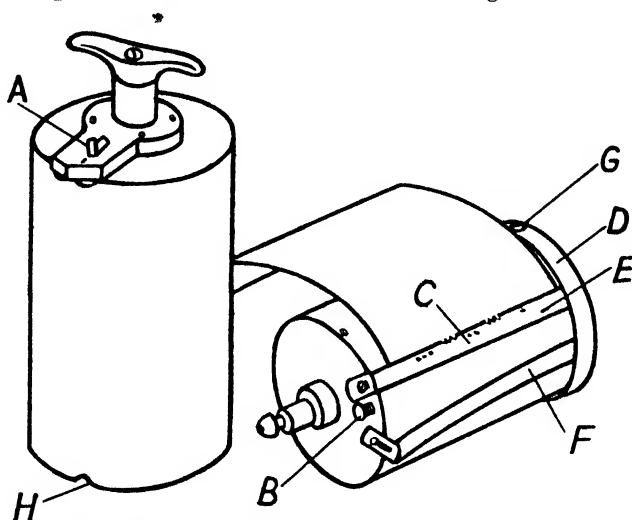
Fig. V.33. Paper-holder of "Cambridge" Polarograph.

pulling gently on the handle, the cover slides off, but is prevented from being drawn right off by a safety bar, into the end of which the drum catch engages.

3. *Recording the Polarogram.* The cover of the paper-holder is pushed in, but is not pressed right home, so that the drum may be rotated inside the cover. This may be done without danger of fogging the paper if the safety bar is left projecting about $\frac{1}{8}$ in. The lamps are switched on, so that a white, general illumination appears on the scale. Having made sure that the potentiometer reads zero, the drum is coupled to the potentiometer by pushing in knob T. The lamps are then switched off and the cover is withdrawn to its fullest extent. The motor is then switched on, followed by the lamps, when recording commences. When this has proceeded to the desired extent, the lamps are switched off and the motor is then stopped.

To ensure the correct functioning of the instrument, the polarogram of the cadmium test solution (p. 450) should be recorded before proceeding with the examination of other solutions. After diluting the 1,000 mg. per litre solution and deoxygenating as previously described, the record is taken and should be allowed to proceed to about 1 volt, when it is stopped. With the mercury still flowing, the capillary is rinsed and the tip immersed in distilled water. The cell is rinsed out and refilled with a half-strength test solution, prepared from 5 ml. of concentrated cadmium solution instead of the 10 ml. previously employed. The sensitivity is increased to 1/10 and another record is taken. It is not necessary to reload the camera; one of the procedures described in the following section may be used.

4. *Multiple Recording.* Several records may be taken upon a single sheet of photographic paper. There are two ways of doing this; the first gives records on the original squared background, while the second produces separate records on previously unexposed portions of the paper. In either case, the first step is to push in the cover of the paper-holder until the safety bar projects about ⅛ in. and then to turn on the lamps.

(1) The dials of the potentiometer and camera are returned to zero. By means of knob N the spot of light is brought to 15 divisions on the scale (assuming the previous record to have been taken with an initial adjustment of 5 divisions). The record is then taken in the usual way, except that the ground lamp switch is pushed *upwards*. This cuts off the white background light and replaces it with a "safe" light which does not affect the photographic paper, so that the original background is unchanged.

(2) Knob T is pulled out and turned until the setting of the camera dial is approximately one division greater than that reached in the previous recording. The *paper indicator*, which is fitted to the camera dial, enables this to be done with ease. The indicator is a small pointer which remains stationary when the dial is rotated in such a manner as to increase the reading, but which moves with the dial when the latter is rotated in the reverse direction. It thus shows automatically the extent of the paper which has been exposed. Thus, in recording the polarogram of the cadmium test solution described above, the camera dial will be found to be 10 if the recording was stopped at 1 volt. Accordingly the dial is set at 11 for recording the next polarogram. Having returned the potentiometer dial to zero and locked it to the camera drum by pushing in knob T, the new record is taken in the usual way with the white background light.

5. *Damping and Counter-current Controls.* The damping of the galvanometer (see p. 447) is controlled by rheostat J. When it is desired to disconnect the condenser, the battery switch is pushed backwards away from the operator.

When working at low concentration and consequent high sensitivity, the *condenser current* due to the charging of the mercury drops (see p. 436)

increases noticeably with the applied voltage, and the portions of the curve before the foot and beyond the crest of the wave are inclined to the voltage axis instead of being approximately parallel to it.   Rheostat K enables a small "counter-current," which is proportional to the voltage being applied to the dropping electrode system, to flow through the galvanometer.   This current, which acts in opposition to the main flow, is adjusted to balance out the "condenser current" effect.   For full sensitivity, K should be set at between 5 and 10 divisions; for sensitivities of less than half, the setting should be zero.
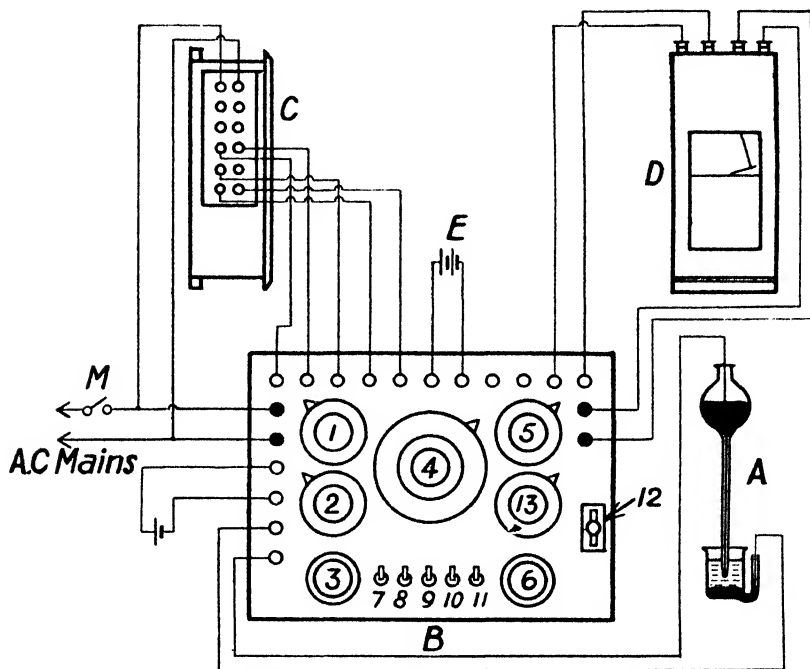
Fig. V.34.   Electrical Connections of the "Tinsley" Ink Recording Polarograph.

Manipulation of the "Tinsley" Ink Recording Polarograph

1. *Preliminary Adjustments.* The apparatus consists of four units (fig. V.34).   These are: dropping mercury electrode assembly A; operating unit B; amplifier C; and ink recorder D.   Power is drawn from the A.C. mains and from 4 volt accumulator E.   An additional 2 volt accumulator is used only when electrical zero setting is required.

Having suitably arranged the apparatus, electrical connections are made. Switches 7–11 inclusive should be in the "off" position.   The dashpot of the recorder is filled with special oil supplied with the instrument and the pen is then inked.

After closing A.C. supply switch M, the apparatus should be left for about

2 minutes to allow the thermal delay switch within the amplifier to close. If the amplifier is switched off, a similar period should be allowed to elapse before switching on again.

Control key 12 is moved to the "off" position and mains switch 11 is turned on. The pen should then commence to draw a line at zero; if not, correction should be made by means of the adjuster, which is in front of the dashpot. On temporarily disconnecting one of the recorder input leads, no alteration in the zero setting should occur.

2. *Standardising.* Mains switch 11 still being on, battery switch 8 is now closed. Control key 12 is moved to the "Standardise" position, when the pen should move across the chart. By means of standardising rheostat 5, the deflection is adjusted until the pen draws a line at full scale. Switch 11 and the control key are then returned to the "off" position. The setting should be examined daily, and may be checked occasionally as desired.

3. *Recording the Polarogram.* The main potentiometer 4 and the roll chart of the recorder should both be set at the voltage at which it is desired to start the record. For example, if it is desired to commence at zero applied E.M.F., the potentiometer should be rotated clockwise a few divisions past zero and then returned until exactly at zero. By this means, any backlash in the gears is taken up. The recorder chart is set in a similar manner. By means of the right-hand knurled knob, the chart is moved forward past zero and is then returned to the latter setting.

For verifying the functioning of the apparatus, the polarogram of the cadmium test solution (p. 450) may be recorded. Having diluted and deoxygenated the cadmium test solution as previously described, range switch 3 is set to its maximum of 200 microamperes (i.e. to the position of lowest sensitivity) and damping (see p. 447) is applied by switching on 7. Main potentiometer 4 and the recorder chart are then set at zero as described above, and control key 12 is placed in the "test" position. On closing mains switch 11, recording should commence. The cadmium wave will be small; as soon as its shape is discernible, range switch 3 should be adjusted so that the record occupies practically the whole width of the chart. A similar procedure is used in the examination of unknown solutions. Damping may be used as required.

4. *Use of Electrical Zero Setting.* This control is used for suppressing a large wave, so that a second, smaller wave may be examined at increased sensitivity (see p. 447). With due regard to polarity, a 2 volt accumulator is connected to the terminals marked "Zero Setting Battery." Having set controls 1 and 2 to zero, switch 10 is closed. Control 2 should be rotated to division 9; the direction and magnitude of the zero setting are then adjusted by operating control 1. Subsequent operation of control 2 provides a fine adjustment for the setting. If damping additional to that provided by switch 7 is required, extra condensers may be connected to

the terminals provided.   The setting should be checked if the sensitivity is altered.

The "condenser current" effect which occurs when working with low concentrations (see p. 436) may be eliminated by closing condenser current switch 9 and operating control 6 until the correct setting is obtained.   Fine adjustment is provided by control 13.   This procedure may alter the zero; if so, the latter is returned to its original position by the electrical method of setting, as described above.

INTERPRETATION OF THE POLAROGRAM

1. Measurement of Wave Height. In performing this operation it is immaterial whether the average, the maximum, or the minimum of the galvanometer oscillations is used, provided that the same convention is

(a)   (b)   (c)
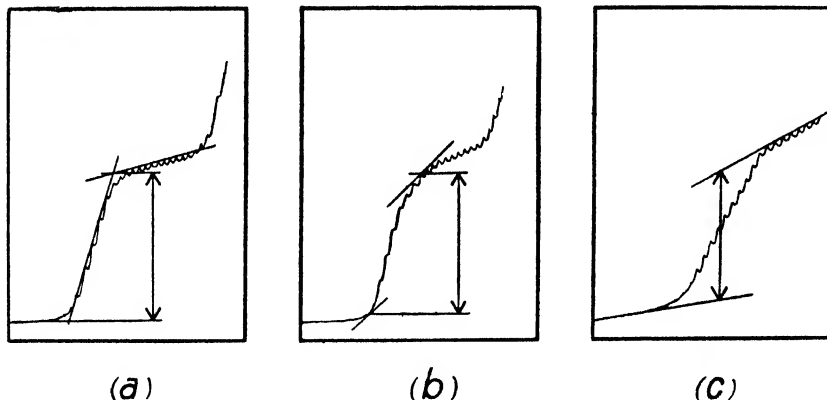
Fig. V.35.   The Measurement of Wave Height.
(a) "Intersection" method; (b) "45° Tangent" method; (c) "Extrapolation" method.

adhered to throughout.   With automatically recorded curves it is definitely advantageous to select the maximum or the minimum, since this facilitates the drawing of the tangents to the curves.   Use of the maximum is the usual convention.

The more usual methods of measurements are as follows:

(a) *"Intersection" Method.* A tangent is drawn to the portion of greatest slope to cut the tangents to the plateaux at the crest and foot of the wave. As shown at (a) in Fig. V.35, the wave height is taken as the vertical distance between the points of intersection of the tangents.

(b) *"45° Tangent" Method.* Tangents are drawn at 45° to the axes to touch the rounded portions of the wave near the crest and foot respectively. The vertical distance between the points of contact is taken as the wave height, as shown at (b) in fig. V.35.

(c) *"Extrapolation" Method.* As in method (a), tangents are drawn to the plateaux of the curve. A vertical is then drawn through the mid-point of the rising portion of the wave (i.e. at a point corresponding with the half-wave potential—see p. 437) to cut the two tangents, as shown at (c) in fig. V.35. The intercept on the vertical is taken as the wave height.

All of the above methods are somewhat arbitrary. Hence it is desirable to select the one most suited to the form of the waves given by the substance to be determined, and to use that method consistently. In general, the particular method adopted does not then greatly affect the results. An exception occurs in the determination of substances present in very low concentration (*ca.* 1 mg. per litre), when a high galvanometer sensitivity has to be used. In such cases, the "extrapolation" method, which is particularly useful for measuring waves which depart from ideal shape, should always be used.

2. Methods of Calibration. The Ilkovic equation (p. 437) indicates that, by measuring all other factors involved, the concentration of the wave-forming substance can be calculated absolutely. Though the theoretical aspects of this are important, such a procedure could hardly be regarded favourably in practical analysis. Accordingly, the concentration of the test solution is usually determined comparing the wave height with those given by a suitable reference solution. Examples of such "comparison" methods are:

(a) *Construction of a Standard Calibration Curve.* This is the most general of these "comparison" methods. A supporting electrolyte, suitable both in nature and concentration, having been chosen, a series of solutions containing known amounts of the substance to be determined is prepared, so that the range of concentrations likely to be encountered in the actual analyses is covered. These are polarographed and the measured wave heights are plotted against the corresponding concentrations. To analyse a solution of unknown concentration, the wave height is determined under the same conditions, and the concentration is then read off from the calibration curve, which should be linear except in certain cases where the Ilkovic equation does not hold.

(b) *Method of Standard Addition.* When a few isolated determinations have to be made, this method is useful. The wave height given by the solution of unknown concentration is measured, a known volume of a standard solution of the same substance is added, and the wave height is measured once more. From the two measurements the concentration originally present may be calculated. Traces of nickel in cobalt salts have been determined in this manner.*

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* Lingane and Kerlinger, *Ind. Eng. Chem.* (Anal. Edn.), 1941, **13**, 77; this paper also gives the method of calculation.

(c) *"Pilot Ion" Method*.* This procedure is also known as the *internal standard* method. To the unknown solution is added a known amount of a wave-forming substance which is *different* from the substance being determined. On polarographing, *two* waves, one due to each substance, are obtained, and their respective heights are measured. Under identical conditions the wave heights obtained with equimolar concentrations of the two substances bear a constant relation to one another. If this relationship, the concentration of *one* of the substances in the test solution, and the respective heights of the waves obtained are known, then the concentration of the second substance may be calculated. For example, a solution containing nickel may be analysed by adding a known concentration of cadmium, and then measuring the heights of the two waves.† If the concentration of the added reference substance (cadmium) in the test solutions is kept constant, it is possible to construct a calibration curve from which the concentration of the second substance (nickel) may be read off directly from the ratio of the two wave heights.

In a similar manner, *several* different substances present in the same solution may be determined by the addition of a single reference substance. Additionally, by incorporating it in the supporting solution, the reference substance may be introduced automatically into the test solution at the required concentration. The method has thus some interesting possibilities.

Another capacity in which the "pilot ion," or internal standard, may serve is in the location of the waves of other substances along the voltage axis. This is sometimes useful when the potential of the mercury pool is uncertain and an external reference electrode cannot readily be applied. Since the half-wave potential of the thallous ion is remarkably constant in solutions diverse in nature, it is sometimes employed for this purpose.

The use of the "pilot ion" method has so far been rather limited, but an interesting application of the principle to the analysis of brass plating has been described.‡

* Forche, *Mikrochem.*, 1938, **25**, 217.
† Cambridge Instrument Co., Ltd., List No. 109, p. 19.
‡ Tyler and Brown, *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15**, 520.

REPRESENTATIVE EXAMPLES OF POLAROGRAPHIC ANALYSIS

From the many hundreds of published methods of analysis by means of the polarograph, a few have been selected to illustrate various points of technique. The determination of small quantities of nitrobenzene in samples of aniline demonstrates the great simplicity in manipulation which is sometimes possible. Since the normal method of performing such a determination involves treatment of the sample with titanous chloride followed by back-titration of the excess of the latter with standard ferric solution, the simplification is obviously quite considerable.

The determination of copper in plant materials, which is dealt with in the second example, is a typical "trace metal" analysis, involving destruction of organic material and removal of interfering substances.

In certain instances, substances which give either poorly defined waves or no waves at all may be determined indirectly. Magnesium, the determination of which is dealt with in the third example, is of this type.

The final two determinations are of metallurgical interest. One is an example of the simultaneous determination of two substances, in this case, iron and zinc, from a single polarogram (see p. 439).

The determination of lead in brasses and bronzes, which is the subject of the fifth example, demonstrates the skilful choice of a supporting solution which not only suppresses an unwanted wave but also enables the form of the polarogram to be improved. Removal of dissolved oxygen by the addition of sodium sulphite (see p. 439) is also rendered possible.

In describing these determinations, it is assumed that a polarographic cell with a mercury pool anode is used.

Many other determinations are described in specialist monographs* and in the literature†; the reader is referred to these for information supplementing that of Table II of pp. 471–472.

Example 1: Determination of Nitro-compounds

In 0·05M sulphuric acid solution, the lower members of the aliphatic series of nitro-compounds give waves the heights of which are proportional to the concentration of the particular nitro-compound present.‡ Certain

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* See, for example, Hohn, "Chemische Analysen mit dem Polarographen" (Berlin, 1937); Kolthoff and Lingane, "Polarography" (New York, 1941); Heyrovsky, "Polarographie" (Vienna).

† Useful bibliographies are published by the Cambridge Instrument Co., Ltd. (London, List No. 109); Leeds and Northrup Co. (Philadelphia, List No. E-94 (1)); E. H. Sargent and Co. (Chicago, 1941). The polarography of organic compounds is discussed by Müller, *Chem. Reviews*, 1939, **24**, 95; this paper contains an extensive bibliography.

‡ de Vries and Ivett, *Ind. Eng. Chem.* (Anal. Edn.), 1941, **13**, 339.

aromatic nitro-compounds were investigated much earlier, mainly by Japanese workers.* Of these, nitrobenzene, the polarographic behaviour of which was first investigated by Shikata,† is of special interest owing to its technical importance, and is the subject of the example chosen.

Application to the Determination of Small Amounts of Nitrobenzene in Aniline‡

The technique described is an improvement of method worked out by Novak§ and gives smooth curves from which amounts of nitrobenzene of the order of 0·025% may be determined readily and accurately.

PRINCIPLES. The sample is polarographed after treatment with a small volume of hydrochloric acid containing a trace of nigrosine. The wave height is proportional to the nitrobenzene content of the sample, which is read off from a calibration curve.

REAGENTS.

Hydrochloric acid/nigrosine solution. Dissolve 0·1 g. of nigrosine in 100 ml. of concentrated hydrochloric acid.

METHOD. 2 ml. of the sample are measured into a small stoppered tube and 0·5 ml. of hydrochloric acid/nigrosine solution is added. The mixture is then shaken thoroughly until the fumes initially produced dissolve completely. When cold, the liquid is transferred to a 2 ml. polarographic cell (see p. 444) and the polarogram is plotted or recorded over the range 0–1·4 volts. The nitrobenzene wave commences at approximately 0·4 volt. To measure the wave height, the "45° tangent" method is used (see p. 457) and the percentage of nitrobenzene in the sample is read off from a calibration curve, which is constructed as below.

Samples of aniline containing approximately 0·01, 0·03, and 0·05% by weight of nitrobenzene respectively are prepared by mixing known weights of the pure substances (see Note). These samples are in turn treated as above, and the measured wave heights are plotted against the corresponding percentages of nitrobenzene. Since the wave heights are proportional to the percentage of nitrobenzene contained in the sample, the three points should lie on a straight line passing through the origin.

NOTE. Commercial "pure aniline" contains traces of nitrobenzene, which may be removed by distilling a large quantity and collecting the distillate when the latter exhibits the correct refractive index for aniline. The sample is then redistilled at 150 mm. pressure, and should exhibit no nitrobenzene wave when tested as above.

* See, for example, Shikata, *J. Agr. Chem. Soc. Japan*, 1925, **1**, 533; Shikata and Watanabe, ibid., 1928, **4**, 924; Shikata and Hozaki, *Mem. Coll. Agr. Kyoto Imp. Univ.*, 1931, **17**, 1, 21.
† Shikata, *Trans. Faraday Soc.*, 1925, **21**, 42.
‡ Haslam and Cross, *J.S.C.I.*, 1944, **63**, 94.
§ Novak, *Collection Czechoslovak Chem. Commun.*, 1939, **11**, 573.

Example 2: Determination of Copper

Copper is readily reduced at the dropping mercury cathode. In the presence of ammonia, pyridine, chloride, and certain other complex-forming substances, a double wave is formed, indicating stepwise reduction by way of the cuprous state. In non-complex-forming media, e.g. sulphuric or nitric acid solutions, a single wave is obtained.* A single wave is also obtained in sodium potassium tartrate and in citrate solutions.† Owing to the ease of reduction (the half-wave potentials referred to the saturated calomel electrode range approximately from $+0.05 - -0.6$ volt, according to the nature of the supporting solution) copper may be estimated in the presence of a variety of other ions which are reduced only at higher potentials. Ferric iron interferes, but may be eliminated quantitatively by precipitation as the hydroxide, as in the example chosen.

Application to the Determination of Copper in Plant Materials‡

PRINCIPLES. Organic matter is destroyed by wet ashing, thus avoiding loss of copper. Iron is removed by precipitation with ammonia and filtered off. After evaporation to dryness, the residue is taken up in a supporting solution containing acid sodium citrate, and the height of the copper wave is determined after eliminating dissolved oxygen. The addition of a trace of acid fuchsin eliminates any tendency to form irregularities ("maxima") on the polarogram. The limit of the method is 0.2 μg. of copper per millilitre of supporting solution or 0.0002% of copper in a 1 g. sample of plant material.

REAGENTS.

1. 60% *perchloric acid.*

2. *Supporting solution.* Mix equal volumes of $0.5M$ sodium hydroxide and $0.5M$ citric acid.

3. 0.05% *acid fuchsin solution.*

4. *Standard copper solution.* Dissolve 0.3928 g. of copper sulphate ($CuSO_4.5H_2O$) in water and make up to 100 ml. 1 ml. of this solution contains 1 mg. of copper. Dilute as required.

METHOD. The sample ($0.5–2.0$ g.) is weighed into a 30 ml. micro-Kjeldahl flask and 5 ml. of concentrated nitric acid are added. After heating until brown fumes are evolved, 1 ml. of concentrated sulphuric acid is introduced and the mixture heated until all the nitric acid is eliminated and charring begins. 1–2 ml. of 60% perchloric acid is now added and the heating continued until the liquid is colourless or pale yellow, the excess of perchloric acid being driven off. After dilution to 15–20 ml. a slight excess of ammonia

* Lingane, *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15**, 583.
† Suchy, *Collection Czechoslovak Chem. Commun.*, 1931, **3**, 354.
‡ Reed and Cummings, *Ind. Eng. Chem.* (Anal. Edn.), 1941, **13**, 124.

is added, the solution boiled for a minute, filtered, and the filter washed with slightly ammoniacal water. The filtrate and washings are evaporated to dryness; the residue is taken up in 9 ml. of supporting solution and 1 ml. of acid fuchsin solution. A portion of the solution is transferred to the polarographic cell and dissolved oxygen is removed by bubbling nitrogen through the liquid (see p. 450). The polarogram is plotted or recorded and the height of the copper wave is measured, using the "intersection" method (see p. 457). A blank determination is made, following the above instructions, but omitting the plant material. In a similar manner, a series of wave heights is obtained by adding known amounts of copper in place of the plant material. The measured wave heights are plotted against the corresponding amounts of copper added and a calibration curve (see p. 458), allowing the copper content of a sample to be read off directly, is thus obtained.

NOTES.

(i) Wet destruction of organic material is essential for the success of the procedure.* Dry ashing gives very low results.

(ii) The construction of the calibration curve in the above manner compensates for the traces of copper in the reagents. Once this curve has been constructed using a given batch of reagents, all that is necessary is to measure the wave height given by the solution from the sample. As a precaution, it is wise to check occasionally by adding a known amount of copper.

(iii) An ingenious application of the acid citrate supporting solution is in the determination of copper in copper-containing proteins.† The metal may be extracted completely from the protein by treatment with dilute acids, and is not adsorbed on the residual protein when the pH is raised to 4 by the addition of alkali. Accordingly, copper may be determined in the dried protein material by extracting with $0.5M$ citric acid solution, adding an equal volume of $0.5M$ sodium hydroxide solution, filtering if necessary, and determining the height of the copper wave. The need for destroying organic material is thus eliminated.

Example 3: Determination of Magnesium

The magnesium ion is reduced only with difficulty at the dropping mercury cathode. In solutions containing tetramethylammonium salts, a wave can be obtained, but it is poorly defined and useless for measurement. Further, it occurs at extremely negative potentials (ca. 2·2 volts with respect to the saturated calomel electrode), so that practically all substances which are polarographically reducible might be expected to interfere if present in appreciable quantity. However, indirect determination, involving

* Cf. Bailey and McHargue, *Plant Physiol.*, 1945, **20**, 79.
† Ames and Dawson, *Ind. Eng. Chem.* (Anal. Edn.), 1945, **17**, 249.

compound-formation with a more readily reducible substance, is possible. Advantage is taken of the fact that 8-hydroxyquinoline (oxine), which precipitates magnesium quantitatively under appropriate conditions, is suitably reducible at the dropping mercury cathode. The magnesium-containing solution, freed from interfering metals (see p. 295 for separation) is treated with a solution of this reagent, whereby the magnesium is entirely precipitated. The precipitate is collected and the 8-hydroxyquinoline contained in it is liberated and determined polarographically, thus affording a measure of the magnesium present.* Alternatively, a known amount of the reagent may be added to the magnesium-containing solution; after precipitation is complete, the excess of 8-hydroxyquinoline is determined. The amount consumed in precipitating the magnesium, and hence the amount of the metal itself is thus determined.† In both cases, a buffered supporting solution is used, since the wave-system of 8-hydroxyquinoline is considerably affected by the pH of the medium. This effect is common in the polarography of organic and other compounds where the hydrogen ion is involved in the electrode-mechanism.‡

Application to the Determination of Magnesium in Mouse Epidermis§

PRINCIPLES. The material is ashed to destroy organic matter, the ash dissolved in hydrochloric acid, and the magnesium precipitated with 8-hydroxyquinoline. The precipitate is collected and treated with hydrochloric acid; the 8-hydroxyquinoline thus liberated is determined polarographically, and is proportional to the magnesium present. Quantities of magnesium down to less than 0·1 mg. may thus be determined to within ±3%.

REAGENTS.

1. *0·1N hydrochloric acid.*

2. *2N ammonium chloride solution.*

3. *6N ammonium hydroxide.*

4. 1% *8-hydroxyquinoline in* 95% *alcohol solution.*

5. *Wash solution.* Saturate 95% alcohol with magnesium hydroxyquinolinate. Filter just before use through a sintered glass filter, the mat of which is covered with a layer of asbestos.

6. *Buffer solution.* Dissolve 1,194 g. of disodium phosphate (Na_2HPO_4. $12H_2O$) and 454 g. of monopotassium phosphate (KH_2PO_4) in water and dilute to 1 l. A buffer solution of pH 7·6 which is 3·33M with respect to both salts is thus obtained.

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* Carruthers, *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15**, 412.
† Stone and Furman, ibid., 1944, **16**, 596.
‡ See, for example, Langer, *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 283; Muller, *J. Chem. Education*, 1941, **18**, 320; Stock, *J. Chem. Soc.*, 1944, 427.
§ See Note (i), p. 466.

7. *Supporting solution.* Prepare as required. Dilute 2·7 ml. of 1% gelatin solution to 100 ml. with the buffer solution.

8. *Diluting solution.* Prepare as required. To 2 ml. of 1% gelatin solution add 30 ml. of 0·1N hydrochloric acid and dilute to 100 ml. with the buffer solution.

METHOD. The sample (0·1–0·4 g.) is weighed into a silica crucible and ignited at 450° C. in a muffle furnace until destruction of organic material is complete. The ash is dissolved in 1 ml. of 0·1N hydrochloric acid and transferred to a 50 ml. Pyrex beaker. The crucible is washed with four 2 ml. portions of water, adding the washings to the beaker. 1 ml. of 2N ammonium chloride and 0·5 ml. of 6N ammonium hydroxide are added, the solution is heated to 96°–100° C., and 0·7 ml. of hydroxyquinoline solution is introduced dropwise, with stirring. After allowing to cool, the contents of the beaker are reheated to or near boiling and a further 0·7 ml. of reagent is added. The procedure is repeated for a third portion of reagent. After standing for 1 hour at room temperature, the liquid is sucked off, using a medium-porosity filter-stick, the mat of which is covered with a thin layer of asbestos. The precipitate is washed with two 2 ml. portions of wash solution and the beaker containing the filter-stick and precipitate is dried at 105° C. for 30 minutes. After cooling, 3 parts of 0·1N hydrochloric acid (see Note (ii)) are added. The precipitate is dissolved by gentle warming and stirring, dislodging particles from the walls by means of the filter-stick, and ensuring that the lower part of the latter is well rinsed. 7 parts of supporting solution are then added, the solution well mixed, and filtered through a dry paper to remove asbestos. A portion of the filtrate is transferred to a 2 ml. polarographic cell, deoxygenated as usual, and the polarogram plotted or recorded. The applied E.M.F. at which the wave height is measured lies between 1·4 and 1·6 volts, increasing with the concentration of 8-hydroxyquinoline in the solution (see Note (iii)). To correct for the residual current, a portion of diluting solution is deoxygenated and polarographed. The value of the residual current is read off at the same applied E.M.F. as above and is deducted. The corrected wave height is applied to a previously constructed calibration curve and the corresponding concentration of magnesium is read off.

The calibration curve is constructed as follows. About 0·5 g. of magnesium hydroxyquinolinate is precipitated, washed thoroughly with water, followed by alcohol, and dried over-night at 105° C. 400 mg. of the dried substance are dissolved in 150 ml. of 0·1N hydrochloric acid and transferred to a 500 ml. graduated flask, rinsing in with several small portions of buffer solution. After adding 10 ml. of 1% gelatin solution, sufficient buffer solution is added to bring up to volume. This solution is $2·29 \times 10^{-3}M$ with respect to magnesium hydroxyquinolinate; 1 ml. is equivalent to 0·0558 mg. of magnesium. By appropriate mixing with diluting solution,

a series of standards containing from 0·0025–0·025 mg. of magnesium per millilitre is prepared. Their wave heights are measured, subtracted from the appropriate values of the residual current, and the corrected wave heights are then plotted against the corresponding concentrations of magnesium.

NOTES.

(i) Iron and calcium are the only elements likely to be found in amounts great enough to interfere in limited quantities of animal tissue. According to Carruthers,* mouse epidermis contains insufficient iron or calcium to render necessary the removal of these metals; for like substances, the separation described on p. 295 is suggested where necessary.

(ii) The volume of acid used to dissolve the precipitate should be such that, after the appropriate addition of supporting solution, the concentration with respect to magnesium hydroxyquinolinate does not exceed $1 \times 10^{-3} M$ and falls within the limits of the calibration curve. The *ratio* of acid to supporting solution is important, since the pH must be maintained at 7·1. If the concentration is too great, it may be reduced without altering the pH by adding diluting solution.

(iii) The wave height is measured at an applied E.M.F. corresponding with the middle of the short diffusion current region. It corresponds to the sum of the heights of two waves, the first of which is small and ill-defined. Beyond this diffusion current region, a third wave, unsuitable for measurement, is formed.

Example 4: Determination of Iron and Zinc

Solutions of *ferric salts* give rise to two waves, corresponding with the reactions $Fe^{III} \rightarrow Fe^{II}$ and $Fe^{II} \rightarrow Fe$ respectively. When a mercury pool anode is used, the wave corresponding with the first reaction starts at zero applied E.M.F., and its half-wave potential is related to the anodic dissolution of mercury, since the latter is attacked by ferric salts. In solutions containing complex-forming ions such as fluoride, oxalate, etc., the half-wave potential is shifted to more negative values and well-defined waves are formed.

Reduction of iron in the *ferrous* state takes place at a much more negative potential; for example, in a potassium chloride or barium chloride supporting solution the half-wave potential is —1·3 volts with reference to the saturated calomel electrode.†

An alternative method of determining iron in the ferrous state is by its *oxidation* at the dropping mercury electrode.‡ The latter then serves as

* *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15**, 412.

† Prajzler, *Collection Czechoslovak Chem. Commun.*, 1931, **3**, 406.

‡ See, for example, Verdier, *Collection Czechoslovak Chem. Commun.*, 1939, **11**, 240; Lingane, *Chem. Reviews*, 1941, **29**, 1.

the anode, and an *anodic wave*, the height of which is proportional to the concentration of ferrous iron in the solution, is obtained.

Zinc forms well-defined waves in a variety of neutral and alkaline solutions, such as those of potassium nitrate, ammonium chloride, or sodium hydroxide. It cannot, however, be determined in solutions of high hydrogen ion concentration, since its wave is masked by hydrogen evolution.* Ammonium oxalate solution has been shown to be useful for determining zinc in the presence of nickel, cobalt, etc.,† while a supporting solution containing ammonium acetate and potassium thiocyanate has proved useful in the determination of small quantities of zinc in plant materials.‡

Application to the Determination of Iron and Zinc in Phosphate Coatings§

This method was developed for the examination of machine-gun links and should be generally applicable to ferrous articles which have been protectively coated by the phosphate process.

PRINCIPLES. A sample of the coating is scraped off and dissolved in oxalic acid solution. After rendering faintly ammoniacal, the solution is polarographed, when two waves, the heights of which are proportional to the concentrations of iron and of zinc respectively, are obtained. A sample weighing a few milligrams thus serves for the simultaneous determination of these two metals.

REAGENTS.

1. *0·75M oxalic acid solution.* Dissolve 95 g. of the dihydrate in water and dilute to 1 l.

2. *0·05% glue solution.* Prepare as required from carpenters' glue.

3. *Standard iron solution.* Dissolve 25 mg. of iron wire in 3 ml. of concentrated nitric acid. Evaporate to dryness, dissolve the residue in a few millilitres of 0·75M oxalic acid, and dilute to 500 ml. 1 ml. of this solution contains 0·05 mg. of iron.

4. *Standard zinc solution.* Dissolve 25 mg. of pure zinc in the minimum quantity of hydrochloric acid and dilute to 500 ml. 1 ml. of this solution contains 0·05 mg. of zinc.

METHOD. A small amount of the phosphate coating is carefully scraped from the unoiled surface of the article. To 4 mg. of the powder are added 20 ml. of 0·75M oxalic acid. When the sample has dissolved, 2 drops of methyl red are added. The solution is then neutralised by dropwise addition of concentrated ammonia; 2 drops of the latter are then added in excess and the solution is diluted to 50 ml. A 5 ml. aliquot is transferred

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* Lingane, *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15**, 583.

† Prajzler, *Collection Czechoslovak Chem. Commun.*, 1931, **3**, 406.

‡ Stout, Levy and Williams, *Collection Czechoslovak Chem. Commun.*, 1938, **10**, 129; Reed and Cummings, *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 489.

§ Knanishu and Rice, *Ind. Eng. Chem.* (Anal. Edn.), 1945, **17**, 444.

to a polarographic cell, 1 drop of 0·05% glue is added, and the solution is deoxygenated by bubbling nitrogen through it for 7 minutes. It is then polarographed over the range 0–1·6 volts. The respective heights of the iron and zinc waves are now measured, using the "extrapolation" method (see p. 458). The concentrations of the two metals are then read off from calibration curves (see p. 458), which may be constructed as follows.

Into three 50 ml. graduated flasks are introduced 1, 5, and 10 ml. respectively of standard iron solution. To each flask are added 20 ml. of 0·75M oxalic acid solution and 2 drops of methyl red, followed by concentrated ammonia, which is added dropwise until the solution is neutral. 2 drops of ammonia are then added in excess and the solutions are diluted to the mark with water. 5 ml. aliquots are successively treated as above, the voltage range for polarographing being from 0–0·5 volt. The wave heights are measured and plotted against the corresponding concentrations of iron. The 3 points and the origin should be collinear. The second calibration curve is constructed in a similar manner, using standard zinc solution and employing a voltage range of from 0–1·6.

NOTE. By taking the samples from a standard area of the articles, different coatings may be compared.

Example 5: Determination of Lead

Well-defined waves of lead may be obtained in neutral, acid, and alkaline solutions. Thus Lingane* recommends 0·1N potassium chloride, 1N hydrochloric or nitric acid, or 1N sodium hydroxide solution for the determination of this metal. Solutions containing tartrate are also suitable.*† Lead produces a wave in solutions containing cyanide. Since copper, which is normally so readily reducible (see p. 462), gives no wave under these conditions, supporting solutions containing potassium cyanide are particularly useful in the determination of small quantities of other metals in the presence of a high concentration of copper, as in the example chosen.

Application to the Determination of Lead in Brasses and Bronzes‡

PRINCIPLES. The sample is dissolved in nitric acid and freed from tin by filtration. An aliquot is then treated with solutions of sodium sulphite, potassium cyanide, sodium hydroxide, and gelatin. Under these conditions, dissolved oxygen is destroyed (see p. 439), so that its removal by passing gas through the solution is unnecessary. The solution is then polarographed and the percentage of lead in the sample is deduced from the height of the wave. To obtain the best results, the amount of cyanide in the solution has to be controlled.

* *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15**, 583.
† Suchy, *Collection Czechoslovak Chem. Commun.*, 1931, **8**, 354.
‡ Milner, *Analyst*, 1945, **70**, 250; ibid., p. 335.

When the sample contains more than 1% of manganese, interference occurs owing to the precipitation of manganese hydroxide. By addition of ammonium citrate, this interference may be prevented.

REAGENTS.

1. *Sodium sulphite solution.* Dissolve 50 g. in 100 ml. of water.

2. *Potassium cyanide solution.* Dissolve 13 g. in 100 ml. of water.

3. 10*N* *sodium hydroxide solution.*

4. 0·2% *gelatine solution.*

5. *Ammonium citrate solution.* Dissolve 100 g. of citric acid in 50 ml. of water and treat with 100 ml. of concentrated ammonia.

6. *Standard lead solution.* Dissolve 4·578 g. of lead acetate $(CH_3COO)_2$ $Pb.3H_2O$ in water, add a few drops of acetic acid, and dilute to 500 ml. 1 ml. of this solution contains 5 mg. of lead.

METHOD. If the sample contains more than 1% of lead, 1 g. is dissolved in a mixture of 5 ml. of concentrated nitric acid and 10 ml. of water. When less than 1% of lead is present, a 2 g. portion is used and the amount of nitric acid is increased to 10 ml. After boiling off nitrous fumes, the solution is cooled and made up to 50 ml. To remove metastannic acid, a portion of the solution is filtered through a dry Whatman No. 40 paper and collected in a dry beaker. 10 ml. of the filtrate are pipetted into a second dry beaker and 10 ml. of sodium sulphite solution added from a burette. In the absence of more than 1% of manganese, 8 ml. of potassium cyanide solution are now added from a second burette. (If a 2 g. portion of the sample has been taken, the volume of potassium cyanide solution is increased to 16 ml.) Finally, 10 ml. of 10*N* sodium hydroxide and 2 ml. of gelatin solution are added from a third and a fourth burette. After well mixing, a portion of the solution is transferred to a polarographic cell, which is placed in a thermostat at 25° C. (compare p. 438). After 10 minutes, the solution is polarographed over the range 0 – —1·0 volt and the height of the lead wave is measured. The percentage of lead in the sample is then read off from a calibration curve, which may be constructed as follows.

Three 1 g. portions of pure copper are each dissolved in a mixture of 5 ml. of concentrated nitric acid and 10 ml. of water. To the solutions are added 2, 6, and 10 ml. respectively of standard lead solution, corresponding to a lead content of 1%, 3%, and 5% respectively of lead in a 1 g. sample. Nitrous fumes are then boiled off. After cooling, the solutions are each diluted to 50 ml., filtered, treated with the appropriate quantities of the various reagents, and polarographed. The wave heights are measured and plotted against the corresponding percentages of lead. The points should lie on a straight line passing through the origin.

Since different volumes of reagents are used when the percentage of lead in the sample does not exceed 1%, a separate calibration curve should

be constructed in a similar manner. 2 g. portions of pure copper are dissolved in a mixture of 10 ml. of concentrated nitric acid and 10 ml. of water, and amounts of standard lead solution (diluted for ease of measurement) to correspond to 0·2%, 0·6%, and 1·0% of lead in a 2 g. sample are introduced. The procedure is then as before.

For samples containing more than 1% of manganese, the appropriate weight is treated normally up to and including the addition of sodium sulphite solution. Before adding the appropriate quantities of the remaining reagents, 2 ml. of ammonium citrate are added. The determination is then completed in the usual way, calibration curves being constructed to allow for the dilution caused by the addition of the extra reagent.

NOTES.

(i) The reagents should be added in the order indicated. They should be measured accurately, since the final solution is not made up to a fixed volume in a graduated flask, but is used as such.

(ii) Apart from manganese, all other elements normally occurring in brasses and bronzes are without interferences.

TABLE II

FURTHER EXAMPLES OF POLAROGRAPHIC ANALYSIS

Analysis	*References*
Determination of Metals	
Aluminium: in magnesium alloys.	Gull, H. C., *J. Soc. Chem. Ind.*, 1937, **56**, 177. Gellor, B. A., and Zan'ko, A. M., *Zavodskaya Lab.*, 1939, **8**, 1030; 1940, **9**, 513. Semerano, G., *Mikrochemie ver. Microchim. Acta.* 1938, **25**, 192.
Antimony: in organic compounds and biological samples. in lead alloys.	Page, J. E., and Robinson, F. A., *J.S.C.I.*, 1942, **61**, 93; see also Lingane, J. J., *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15**, 583. Kraus, R., and Novak, J. V., *Die Chemie*, 1943, **56**, 302; Hourigan, H. F., *Analyst*, 1946, **71**, 524.
Bismuth: in organic compounds and biological samples.	Page, J. E., and Robinson, F. A., loc. cit.; see also Lingane, J. J., loc. cit.
Cadmium: in zinc alloys. in dust and fumes in air.	"Polarographic and Spectrographic Analysis of High-purity Zinc and Zinc Alloys for Die-casting," B.S.I. 1225: 1945. Silverman, L., *Chemist-Analyst*, 1946, **35**, 53.
Calcium: indirectly by means of pic-rolonic acid. in presence of other alkaline earth metals.	Cohn, G., and Kolthoff, I. M., *J. Biol. Chem.*, 1943, **147**, 705. Zlotowski, I., and Kolthoff, I. M., *J. Phys. Chem.*, 1945, **49**, 386.
Manganese: as pyrophosphatoman-ganiate.	Kolthoff, I. M., and Watters, J. I., *Ind. Eng. Chem* (Anal. Edn.), 1943, **15**, 8.
Nickel: in cobalt salts. in steel and nickel ores. in aluminium alloys. in copper alloys.	Lingane, J. J., and Kerlinger, H., *Ind. Eng. Chem.* (Anal. Edn.), 1941, **13**, 77. West, P. W., and Dean, J. F., *Ind. Eng. Chem.* (Anal. Edn.), 1945, **17**, 686. Kolthoff, I. M., and Matsuyama, G., *Ind. Eng. Chem.* (Anal. Edn.), 1945, **17**, 615. Milner, G. W. C., *Analyst*, 1945, **70**, 468.
Potassium: in various inorganic and organic materials. in soils.	Weaver, J. R., and Lykken, L., *Anal. Chem.*, 1947, **19**, 372. Okáč, A., *Bodenkunde u. Pflanzenernähr.*, 1945, **36**, 37.
Sodium: in biological materials. in aluminium alloys.	Carruthers, C., *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15**, 70; see also Weaver, J. R., and Lykken, L. loc. cit. Smart, R., *J.S.C.I.*, 1943, **62**, 213; see also Urech, P., and Sulzberger, R., *Helv. Chim. Acta*, 1944, **27**, 1074.
Tin: in foods and biological materials. with lead and zinc, in phenol. in zinc alloys.	Godar, E. M., and Alexander, O. R., *Ind. Eng. Chem.* (Anal. Edn.), 1946, **18**, 681. Wilson, H. N., and Hutchinson, W., *Analyst*, 1947, **72**, 149. Hawkings, R. C., Simpson, D., and Thode, H. G., *Can. J. Research*, 1947, **25B**, 322.

Analysis	References
Vanadium: in organic substances.	Page, J. E., and Robinson, F. A., *Analyst*, 1943, **68**, 269.
in steels.	Lingane, J. J., and Meites, L., *Anal. Chem.*, 1947, **19**, 159.
Determination of Acid Radicals	
Bromide (also other halides, cyanide, sulphide, etc.).	Kolthoff, I. M., and Miller, C. S., *J. Amer. Chem. Soc.*, 1941, **63**, 1405.
Nitrate:	Kolthoff, I. M., Harris, W. E., and Matsuyama, G., *J. Amer. Chem. Soc.*, 1944, **66**, 1782.
in sodium nitrite.	Haslam, J., and Cross, L. H., *J.S.C.I.*, 1945, **64**, 259.
Pyrophosphate: indirectly by means of cadmium.	Cohn, G., and Kolthoff, I. M., *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 886.
Determination of Organic Substances	
Acrolein: in the presence of formaldehyde and acetaldehyde.	Moshier, R. W., *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15**, 107.
Alkaloids.	Kirkpatrick, H. F. W., *Quart. J. Pharm. Pharmacol.*, 1945, **18**, 245, 338; 1946, **19**, 8, 127, 526; 1947, **20**, 87.
Ascorbic acid: in fruits and vegetables.	Gillam, W. S., *Ind. Eng. Chem.* (Anal. Edn.), 1945, **17**, 217; see also Page, J. E., and Waller, J. G., *Analyst*, 1946, **71**, 65.
in milk.	Perrin, D. R., and Perrin, D. D., *New Zealand J. Sci. Technol.*, 1946, **28A**, 266.
Formaldehyde: in the presence of acrolein and other aldehydes.	Whitnack, G. C., and Moshier, R. W., *Ind. Eng. Chem.* (Anal. Edn.), 1944, **16**, 496.
Maleic acid: in mixtures with fumaric acid.	Warshowsky, B., Elving, P. J., and Mandel, J., *Anal. Chem.*, 1947, **19**, 161.
in succinic acid.	Silverman, L., *Chemist-Analyst*, 1947, **36**, 57.
Nitromethane: in air.	Wilson, H. N., and Hutchinson, W., *Analyst*, 1947, **72**, 432.
Steroid hormones.	Barnett, J., Henly, A. A., and Morris, C. J. O. R., *Biochem. J.*, 1946, **40**, 445; Barnett, J., and Morris, C. J. O. R., *Biochem. J.*, 1946, **40**, 450; Barnett, J., Henly, A. A., Morris, C. J. O. R., and Warren, F. L., *Biochem. J.*, 1946, **40**, 778.
Determination of Miscellaneous Substances	
Oxygen: in sewage.	Ingols, R. S., *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 256.
in sea water.	Giguère, P. A., and Lauzier, L., *Can. J. Research*, 1945, **23B**, 76.
Surface-active substances.	Schwarz, K. E., Schroder, H. J., and v. Stackelberg, M., *Z. Elektrochem.*, 1942, **48**, 6; v. Stackelberg, M., and Schutz, H., *Kolloid-Z.*, 1943, **105**, 20.

AMPEROMETRIC TITRATION

1. General Principles. Besides serving as a means for the direct estimation of a substance by wave height measurements, the dropping electrode may also be used as an indicator of the progress of a reaction, e.g. a titration involving precipitation. It is used thus in the technique of *amperometric titration,** in which the electrical measurements are made solely to locate the end-point and do not enter into the calculation of results.

Titrations which may be carried out by this method usually, but not invariably, involve precipitation reactions. Suppose we have two sub-stances, X and Y, which are separately soluble, but which precipitate one another when their solutions are mixed. In general, X and Y will have different polarographic charac-teristics, as shown in fig. V.36. As depicted, the wave of sub-stance X starts at an applied E.M.F. *a*, while the diffusion current region lies between *b* and *d*. Substance Y is shown as being less readily reduced, so that the diffusion current region is shorter, viz. between *c* and *d*.

A solution of X containing the necessary supporting elec-trolyte is placed in the cell, freed from dissolved oxygen, and an E.M.F. between *c* and *d* applied. A current propor-
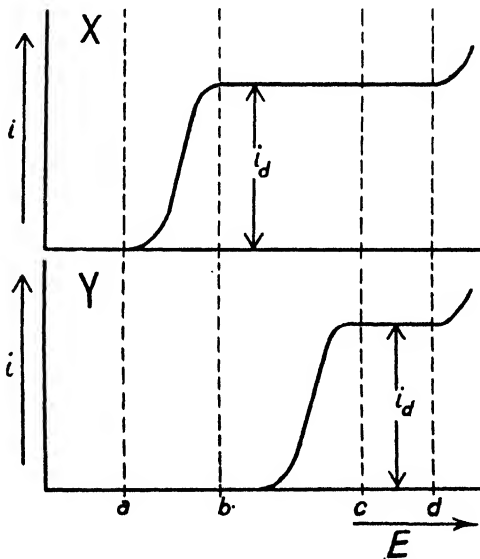
Fig. V.36. Polarographic Waves, showing Diffusion Current Regions.

tional to the concentration of X will flow. Successive amounts of a solution of Y are now added from a burette. Each addition precipitates part of X, so that its concentration, and hence the current, falls propor-tionately until, when all has been precipitated, the current approaches zero. The next addition of Y introduces a new reducible substance into the solution, and as the titration is continued the current rises progressively.

By plotting the current flowing against the corresponding volume of titrant added, two straight lines arranged in the form of a **V** and intersecting

* See Kolthoff and Pan, *J. Amer. Chem. Soc.*, 1939, **61**, 3402, for nomenclature and historical aspects of this technique.
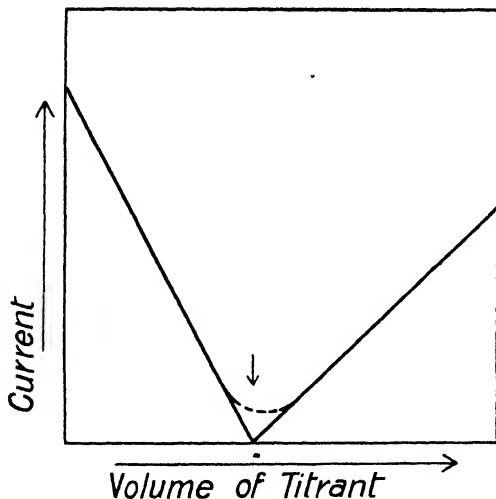
Fig. V.37.   Amperometric Titration Curve of Reducible
Substance Titrated with Reducible Reagent.

to indicate the end-point volume (see fig. V.37) are obtained. Lead ions titrated with potassium dichromate solution at an applied E.M.F. of −1·0 volt (vs. S.C.E.) give a titration curve of this type.

Suppose that a second titration be carried out, the applied E.M.F. being reduced to but slightly in excess of b. Up to the end-point the current decreases as before. At the E.M.F. now being applied, Y is incapable of making any contribution to the current. Hence on continuing the titration the current remains small and virtually constant. The titration curve (fig. V.38) resembles a rough **L** in shape. This is usual when conditions are such that the substance to be determined is reducible, but the titrant is not. When it is the titrant *only* which is reducible (i.e. titration of Y and X at voltage b), a "reversed **L**" curve, depicted in fig. V.39 is obtained.

In practice, owing to the dilution of the test solution by introduction of the titrant, the arms of the titration curve are not quite linear, although the deviation is small if the titrant solution is 10–20 times as concentrated as the test solution. The use of concentrated reagents has the added advantage of introducing but little dissolved oxygen into the system, rendering unnecessary prolonged bubbling with inert gas after each addition. By application of the expression

$$i_{corr.} = \frac{V+x}{V} \, i_{obsd.}$$

where V is the initial volume of the test solution, and $i_{obsd.}$
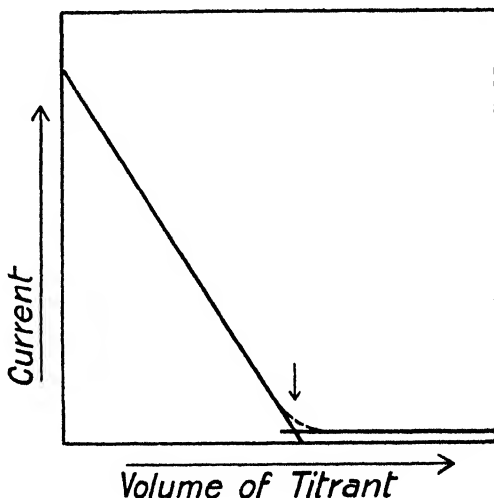
Fig. V.38.   "L"-Type Curve, Obtained when the
Titrant Forms No Wave.

the measured current after adding x ml. of reagent, the current may be corrected for the dilution effect and the arms of the titration curve then become truly linear.

Solubility of the precipitate causes the titration curve to be rounded off at the end-point, as shown by the broken lines in figs. V.37 to V.39. Unless excessive, it does not interfere, since the end-point is located graphically. It is usually sufficient to take a few well-separated readings either side of the end-point and not too close to it, and to draw a pair of straight lines

Fig. V.39. "Reversed L" Curve, Obtained when Only the Titrant Forms a Wave.

through the graphical plot of these readings. To permit accurate location of the end-point it is desirable to correct the current as above.

Another type of titration curve (fig. V.40) is obtained when one of the reactants yields an *anodic* wave (p. 467), whilst the other, under the same conditions of applied E.M.F., etc., yields the more usual cathodic wave. As before, the current decreases as the end-point is approached; having passed the latter, the current again increases, *but its direction is reversed.* The resulting titration curve has the appearance of a single line cutting the volume axis at the end-point, but the two portions of the graph will not necessarily have the same slope. The reaction between ferric salts and titanous chloride gives a curve of this type.

Fig. V.40. Titration Curve of a Substance Forming an Anodic Wave.

2. Scope and Limitations. Owing to its nature, amperometric titration possesses most of the advantages (and disadvantages) associated with polarography. Frequently solutions as dilute as 10^{-3}–$10^{-4}M$ may be titrated, even when but small

volumes are available. "Foreign" salts may be present without interference, and are in fact usually added, while the graphical location of the end-point makes the technique valuable when other methods depending on the natural recognition of the end-point break down.

Since the *change* in current and not its absolute magnitude is all that is required, the characteristics of the capillary, provided they are suitable, do not affect the results. No recalibration is needed should a breakage occur, nor is a thermostat necessary. The process being carried out at an applied E.M.F. which remains fixed, very simple electrical equipment suffices (see below).

Several of the well-known organic precipitants for metals, such as dimethylglyoxime* and α-benzoinoxime,† have been found to give well defined polarographic waves, and these have proved very useful for amperometric titration of nickel, copper, etc.

If substances more readily reducible than the participants in the titration reaction are present, they will of course contribute to the diffusion current. Where the concentration of the interfering substance is large, preliminary chemical separation may have to be resorted to; for lesser amounts, compensation (see p. 447) may obviate the loss of accuracy caused by having to reduce the galvanometer sensitivity. Apart from this, the general difficulties of volumetric analysis, such as slowness of precipitation, co-precipitation, and instrumental errors (compare pp. 130–141 and 158–160) have to be reckoned with.

3. Apparatus and Experimental Technique. Since amperometric titration is carried out at constant applied E.M.F. the simple polarising unit shown in fig. V.28 is adequate, whilst a damped microammeter may be used to measure the current.

When handling moderately large (20–50 ml.) volumes of solution, the cell shown in fig. V.26, *b* is useful. It is advantageous to store the reagent beneath an atmosphere of inert gas so that no dissolved oxygen is introduced during the titration. For dealing with 5–10 ml. of solution a convenient cell can be made from a Pyrex boiling tube (see p. 479).‡

Fig. V.41 illustrates a cell designed to handle less than 1 ml. of solution.§ The tangential arrangement of the gas inlet permits of rapid mixing and elimination of dissolved oxygen without spurting. After completion of titration, the solution chamber may be detached at the lower ground joint and another inserted in its place.

In certain cases, Laitinen and Kolthoff‖ have shown that a rotating platinum micro-electrode may replace the dropping mercury electrode.

* Kolthoff and Langer, *J. Amer. Chem. Soc.*, 1940, **62**, 211.
† Langer, *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 283.
‡ Fill and Stock, *Analyst*, 1944, **69**, 178.
§ Stock, *Analyst*, 1946, **71**, 583.
‖ Laitinen and Kolthoff, *J. Phys. Chem.*, 1941, **45**, 1079; for bibliography up to the end of 1946, see Stock, *Metallurgia*, 1947, **36**, 51.

The correct conditions for carrying out an amperometric titration can be largely deduced from the polarographic characteristics of the substance to be determined and of the proposed reagent. Thus the required applied voltage may be selected and the nature of the supporting electrolyte decided. Preliminary experiments having shown the absence of unforeseen difficulties, the general procedure is as follows:

A known volume of the test solution is placed in the titration cell, which is then assembled, the electrical connections are completed, and dissolved
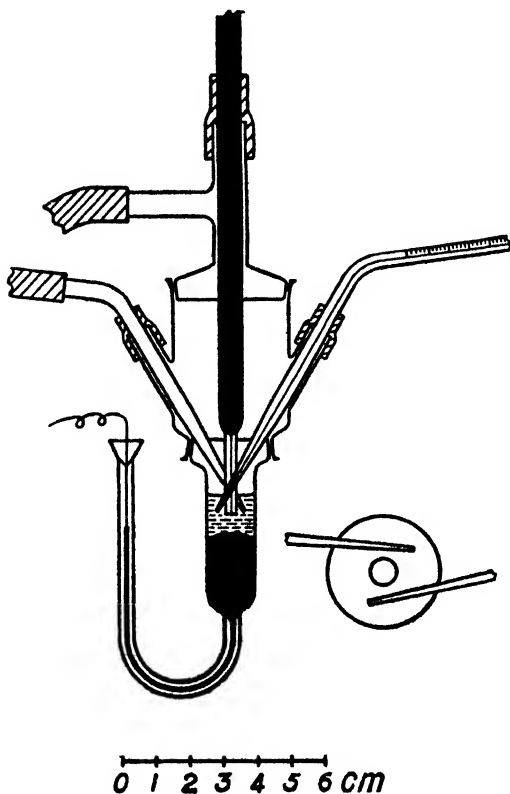
Fig. V.41. Cell for Titrating Volumes of Less than 1 ml.

oxygen is removed by a stream of nitrogen or hydrogen. The applied E.M.F. is then adjusted to the desired value and the initial diffusion current is noted.

A known volume of reagent is run in from a burette, and the gas stream is bubbled through the solution for about 2 minutes to ensure complete mixing and to eliminate traces of oxygen from the added reagent. After stopping the flow of gas, the current and burette reading are both noted. This procedure is repeated until sufficient readings have been obtained to permit the end-point to be determined as the intersection of the two linear parts of the graph.

REPRESENTATIVE EXAMPLES OF AMPEROMETRIC
TITRATION

The technique of amperometric titration using the dropping mercury electrode is of comparatively recent development, and much investigation remains to be done. Two examples have been chosen from the limited number of titrations as yet investigated*; the first demonstrates the sensitivity of the method and the use of organic reagents for inorganic ions. The determination of sulphate, dealt with in the second example, affords a good illustration of the methods of overcoming difficulties such as solubility of precipitate and interference caused by massive amounts of foreign material.

Further examples are listed in Table III of page 482.

Example 1: Titration of Nickel

The amperometric titration of this metal using a solution of dimethylglyoxime as reagent was first reported by Neuberger.† The method was investigated much more fully by Kolthoff and Langer.‡ These workers showed that, under suitable conditions, results accurate to within 0·4% could be obtained with solutions as dilute as $0·001M$ with respect to nickel, and that solutions ten times more dilute could be titrated with a somewhat decreased accuracy. In the example chosen, the small cell described on p. 476 is used, allowing amounts of nickel of the order of a few tenths of a milligram to be conveniently titrated.

PRINCIPLES. The nickel is introduced into an ammonia/ammonium chloride supporting solution and, after deoxygenation, titration is carried out on applied voltage at which both nickel and dimethylglyoxime are reducible. A V-shaped titration curve (see p. 474) is obtained.

REAGENTS.

1. $0·02M$ *dimethylglyoxime solution.* Dissolve 1·161 g. of dimethylglyoxime in 95% alcohol and make up to 500 ml. with the same solvent.

2. *Supporting solution.* Dissolve 2·68 g. of ammonium chloride in 500 ml. of $0·5M$ ammonia. A solution which is $0·5M$ with respect to ammonium hydroxide and $0·1M$ with respect to ammonium chloride is thus obtained. When required for use, add 1 ml. of 3% gelatin solution to 100 ml. of the above.

METHOD. About 1–1·5 ml. of mercury are introduced into the cell (fig. V.42), followed by 5 ml. of supporting solution. By means of a micro-burette, the nickel-containing solution (equivalent to 0·05–1 mg. of nickel) is added. The cell is then assembled and the solution is deoxygenated by bubbling

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* For bibliography up to the end of 1946, see Stock, *Analyst*, 1947, **72**, 291; ibid. *Metallurgia*, 1947, **36**, 51.

† *Z. anal. Chem.*, 1939, **116**, 1.

‡ *J. Amer. Chem. Soc.*, 1940, **62**, 211.

hydrogen or nitrogen for 5 minutes. An E.M.F. of 1·7 volts is applied, the dropping electrode being made the cathode as usual, and the titration is carried out as described on p. 477, adding the dimethylglyoxime solution from a second micro-burette. After correcting for the diluting effect of the added reagent, the titration curve is constructed and the end-point volume is read off.

1 ml. of 0·02M dimethylglyoxime solution $\equiv$ 0·587 mg. of nickel.

NOTES.

(i) Cobalt and copper interfere badly, even when present in small amounts, and must be removed.
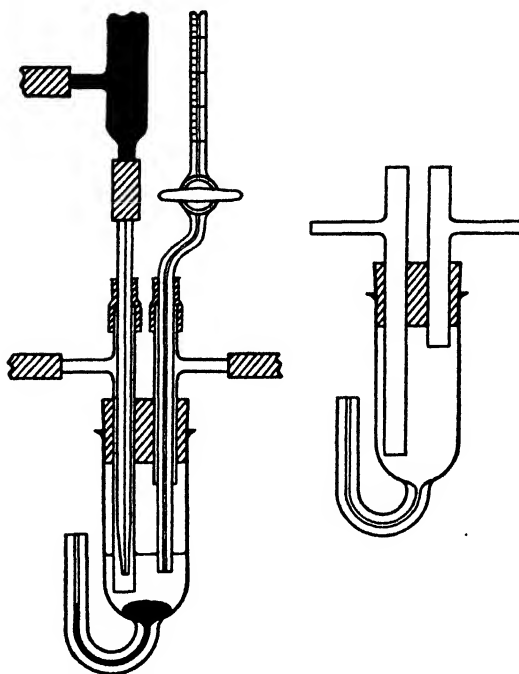
Fig. V.42.   Titration Cell of Simple Construction.

(ii) Aluminium and trivalent iron and chromium are precipitated by the supporting medium and may be present in amounts up to about double that of nickel. Zinc and cadmium may be present in amounts up to that of the nickel, although the accuracy of the titration is somewhat reduced.

Example 2: Titration of Sulphate*

Under suitable conditions solutions as dilute as 0·001M with respect to sulphate may be titrated accurately with lead nitrate solution, the strength

* Majer, *Z. Elektrochem.*, 1936, **42**, 120, 123; Spalenka, *Collection Czechoslovak Chem. Commun.*, 1939, **11**, 146; Kolthoff and Pan, *J. Amer. Chem. Soc.*, 1940, **62**, 3332.

of which should be at least $0 \cdot 01M$. The difficulty due to the appreciable solubility of the precipitated lead sulphate, causing the titration curve to be excessively rounded at the end-point (see p. 475), is overcome by the addition of ethyl alcohol. Indifferent salts, e.g. alkali chlorides or nitrates, tend to increase the solubility of lead sulphate; additionally, potassium salts may cause the precipitation of the double salt $PbSO_4,K_2SO_4$ and hence cause low results. Accordingly, indifferent salt concentrations more than five to ten times greater than that of sulphate should in general be avoided. Even when the presence of a large concentration of indifferent salt is unavoidable, as in the example chosen, it may be possible to perform the titration by selecting the optimum conditions.

Application to the Determination of Residual Sulphate in Precipitated Alumina[*]

PRINCIPLES. The sulphate ion is not reduced at the dropping mercury electrode, but may be titrated amperometrically with a solution of a lead salt, when a "reversed L" curve (see p. 475) is obtained. Lead sulphate is appreciably soluble in aluminium nitrate solution, in which the titration is performed. However, by adding ethyl alcohol, keeping the concentration of aluminium below a certain maximum, and by adjusting the pH of the solution, the titration may be performed satisfactorily.

REAGENTS.
1. 30% *nitric acid.*
2. $0 \cdot 05M$ *lead nitrate solution.*
3. *Ethyl alcohol* (industrial spirit).
4. $0 \cdot 1\%$ *thymol blue solution.*
5. $0 \cdot 1\%$ *methyl orange solution.*

METHOD. To 1 g. of the sample, containing $0 \cdot 5$–4% of sulphate (expressed as SO_3), are added 25 ml. of 30% nitric acid. The mixture is heated until syrupy and a small amount of white residue remains. To this 25 ml. of water are added, and the solution is warmed. It is then transferred to a 50 ml. graduated flask (filtration is unnecessary) and diluted to volume, rendering just acid to thymol blue (but see Note (i)) before finally adjusting to the mark. An aliquot containing less than $0 \cdot 4$ g. of alumina (Al_2O_3) is transferred to the titration cell (fig. V.26, *b*) and 2 volumes of alcohol are added. If necessary, the solution is readjusted until just acid to thymol blue (but see Note (i)). As tream of hydrogen or nitrogen is bubbled through the solution for 10 minutes, an E.M.F. of $1 \cdot 2$ volts applied so that the dropping electrode becomes the cathode, and the lead nitrate solution is added from a 10 ml. burette, passing the gas stream after each addition (see Note (ii)). Not until the end-point has been passed does the galvanometer reading increase appreciably. The current readings are corrected for the diluting

[*] Davies and Key, *Industrial Chemist*, 1943, **19**, 167.

effect of the reagent (see p. 474), and the end-point volume is read off by constructing the titration curve.

1 ml. of $0 \cdot 05M$ lead nitrate solution $\equiv$ 4 mg. of SO_3.

NOTES.

(i) If the aliquot taken for titration is such that after addition of alcohol the solution contains less than $0 \cdot 125$ of Al_2O_3 per 30 ml., then methyl orange should be used in place of thymol blue.

(ii) When the amount of sulphate is small, the gas stream should be passed for 3 minutes after each addition of reagent in order to complete the precipitation.

(iii) Sulphate in boiler water, etc., may be determined by this method, either directly or, if the sulphate concentration is low, after evaporation. Another application is to the determination of sulphur in coke after oxidising the sulphur to the sulphate.*

(iv) If ammonium salts are present, the solution is made alkaline with 20% sodium hydroxide solution and boiled.

* Butenko and Pindas, *Zavodskaya Lab.*, 1940, **9**, 634.

TABLE III

FURTHER EXAMPLES OF MICRO-ANALYSIS BY AMPEROMETRIC TITRATION

Analysis	References
Determination of Metals	
Barium: titration with chromate.	Kolthoff, I. M., and Pan, Y. D., *J. Amer. Chem. Soc.*, 1939, **61**, 3402.
Cadmium: titration with naphtho-quinoline.	Sandberg, B., *Svensk. Kem. Tid.*, 1946, **58**, 197.
Calcium: indirect determination with picrolonic acid and methylene blue.	Cohn, G., and Kolthoff, I. M., *J. Biol. Chem.*, 1943, **148**, 711.
Copper: titration with α-nitroso-β-naphthol. titration with α-benzoinoxime.	Kolthoff, I. M., and Langer, A., *J. Amer. Chem. Soc.*, 1940, **62**, 3172. Langer, A., *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 283.
Iron: titration with 5 : 7-dibromo-8-hydroxyquinoline.	Sandberg, B., *Svensk. Kem. Tid.*, 1946, **58**, 197.
Lead: titration with dichromate.	Kolthoff, I. M., and Pan, Y. D., *J. Amer. Chem. Soc.*, 1939, **61**, 3402.
Palladium: titration with α-nitroso-β-naphthol.	Kolthoff, I. M., and Langer, A., *J. Amer. Chem. Soc.*, 1940, **62**, 3172.
Potassium: titration with sodium dipicrylaminate.	Sandberg, B., *Svensk. Kem. Tid.*, 1946, **58**, 197.
Determination of Acid Radicals	
Chloride: titration with silver nitrate. with other halides in mixtures.	Laitinen, H. A., and Kolthoff, I. M., *J. Phys. Chem.*, 1941, **45**, 1079. Laitinen, H. A., Jennings, W. P., and Parks, T. D., *Ind. Eng. Chem.* (Anal. Edn.), 1946, **18**, 355, 358.
Chromate: titration with ferrous iron.	Kolthoff, I. M., and May, D. R., *Ind. Eng. Chem.* (Anal. Edn.), 1946, **18**, 208.
Cyanide: titration with silver nitrate.	Laitinen, H. A., Jennings, W. P., and Parks, T. D., *Ind. Eng. Chem.* (Anal. Edn.), 1946, **18**, 574.
Fluoride: titration with thorium or lanthanum nitrate.	Langer, A., *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 511.
Phosphate: titration with uranyl acetate.	Kolthoff, I. M., and Cohn, G., *Ind. Eng. Chem.* (Anal. Edn.), 1942, **14**, 412.
Determination of Organic Compounds	
Mercaptans : titration with silver nitrate.	Kolthoff, I. M., and Harris, W. E., *Ind. Eng. Chem.* (Anal. Edn.), 1946, **18**, 161.
Styrene: titration with bromate.	Kolthoff, I. M., and Bovey, F. A., *Anal. Chem.*, 1947, **19**, 498.
α-Tocopherol: titration with gold chloride.	Smith, L. I., Spillane, L. J., and Kolthoff, I. M., *J. Amer. Chem. Soc.*, 1942, **64**, 646.

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5. Leeds and Northrup Co.: List No. E-94 (1) (Philadelphia).

6. Müller, O. H., *Chem. Reviews*, 1939, **24**, 95 (polarography of organic compounds)

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8. Heyrovsky, J., *Collection Czechoslovak Chem. Commun.*, 1938, **10**, 153; 1939, **11**, 98, 667; 1947, **12**, 156 (Bibliographies).

9. Stock, J. T., *Analyst*, 1947 **72**, 291; ibid., *Metallurgia*, 1947, **36**, 51 (amperometric titration).

ELECTRO-DEPOSITION

Introduction

Upon electrolysis between inert electrodes a metal commences to be deposited from its solution when the cathode potential is made slightly more negative than the *reduction potential* of the metallic ion. Differences in reduction potential permit quantitative analysis of a solution containing several metals by performing successive depositions at appropriate cathode potentials taken in increasing order.

Apart from the obvious necessity of obtaining the deposit in a form suitable for weighing, various factors impose limitations upon this method. Obviously, the reduction potential of a particular metal must be less than the potential at which hydrogen evolution commences. Fortunately, hydrogen exhibits a pronounced *overpotential* effect, i.e. is not liberated until a potential higher than that of a reversible hydrogen electrode is applied. The deposition of metals such as zinc and cadmium, which are more electro-negative than hydrogen, is made possible by the existence of hydrogen overpotential.

Another factor of importance in the separation of two or more metals is the change in reduction potential which occurs as the deposition proceeds. Considering the partially deposited metal as an electrode in reversible equilibrium with its ions, its equilibrium potential at 17° C. is given by the general equation

$$E_M = E_0 + \frac{0 \cdot 058}{z} \log_{10}[\text{ion}].$$

During the process of deposition, [ion] decreases, causing E_M to become progressively more negative. For practical purposes, deposition may be taken as complete when the concentration of the metal in the solution has fallen to one-thousandth of its initial value. Under these conditions, E_M changes by $3 \times 0 \cdot 058/z$ volt, e.g. by about 0·2 volt for a univalent ion, during the deposition. Hence the initial reduction potentials of two metals must differ by *at least* this amount for their quantitative separation to be possible.

Complex-formation may permit the separation of metals such as bismuth and copper, which, owing to the proximity of their reduction potentials, are co-deposited from solutions of their simple salts. On addition of potassium cyanide to a solution containing bismuth and copper ions, the latter form a complex and the reduction potential becomes much more negative. That of bismuth suffers little change, so that a separation is then possible.

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Unless the reduction potentials of the ions differ considerably, control of the cathode potential is of great importance in performing separations. In macro-analysis control is frequently achieved by the use of an auxiliary reference electrode. Owing to the small quantities involved, micro-deposition requires but small current densities; this, together with the use of a comparatively large, depolarised anode arranged close to the cathode (as in the Clarke-Hermance and Lindsey-Sand cells), enables the cathode potential to be controlled quite closely by observing the E.M.F. applied to the electrodes. An auxiliary electrode is thus unnecessary.*

Deposition is protracted if the solution is not agitated during the process, since concentration polarisation (compare p. 437) occurs. Stirring, which greatly speeds the analysis, may be effected by bubbling gas through the solution, or, when feasible, by carrying out the deposition at the boiling-point.

Certain metals such as lead and cobalt may be deposited upon the *anode* as the oxide and are weighed in this form. An example of this technique is given on p. 495.

Apparatus and Experimental Technique

1. The Pregl Cell.† This apparatus, which is intended for depositions which can be carried out from boiling solutions (see, however, p. 494) is depicted in fig. V.43, a. The stand incorporates a micro-burner for heating the electrolysis vessel, which is of 16 mm. external diameter and 105 mm. in length. Evaporation during electrolysis is prevented by a reflux condenser filled with cold water. Circulation of water within the condenser and hence cooling efficiency may be improved by forming a bulb near the tip of the condenser, as shown in fig. V.43, b, and inserting a capillary of 1 mm. bore and 50 mm. in length.‡

Deposition takes place upon the outer electrode (fig. V.44, a), which usually acts as the cathode and is a cylinder of platinum gauze. The upper and lower edges each carry three small glass beads, which are fused on. These prevent the gauze from abrasion by the walls of the vessel. A stout platinum wire is welded on, and its extremity (b) is bent over to dip into a mercury cup, thus making electrical contact with the outer circuit. The inner electrode, the upper end of which likewise dips into a second mercury cup, is of stout platinum wire and normally acts as the anode. It is maintained centrally in the cell by the two Y-shaped glass distance pieces which are fused on.

Current may be conveniently drawn from a 4 volt accumulator, employing a circuit including a potentiometer and a milliammeter.

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* For a review of the micro-chemical aspects of electro-deposition, see Lindsey, A. J., *Analyst*, 1948, **73**, 67.

† "Quantitative Organic Micro-analysis" (5th Edition, Vienna, 1947), p. 180.

‡ Bradford and Kirk, *Ind. Eng. Chem.* (Anal. Edn.), 1941, **13**, 64.

Manipulation of the apparatus is as follows.   The cathode is cleaned by successive treatment with hot concentrated nitric acid, water, alcohol, and pure ether.   It is then carefully dried by holding above a flame.   For this purpose, a platinum hook is mounted on the stand (Fig. V.43, *a*). Alternatively, the Lindsey drying apparatus (see p. 491) may be used.   After

(a)   *(b)*
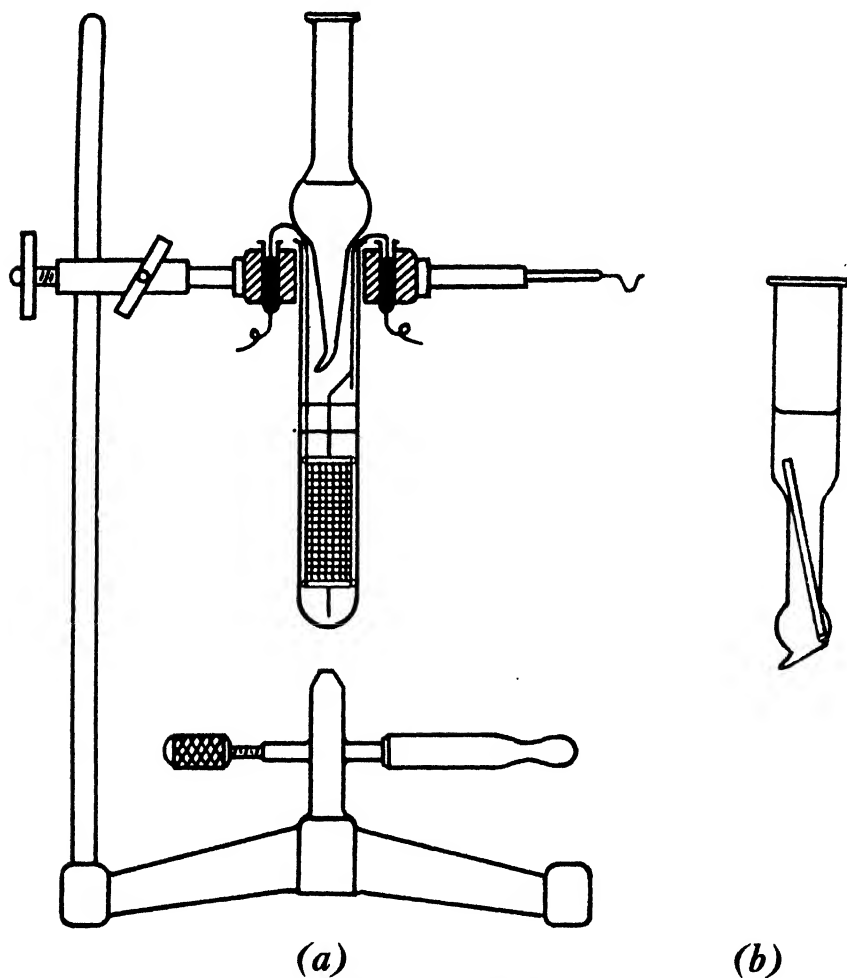
Fig. V.43.   The Pregl Cell.

cooling for 5 minutes the electrode is suspended from the stirrup hook of the micro-balance and weighed 5 minutes later, observing the precautions given on pp. 14–17.

The volume of solution to be electrolysed should be such that the surface is from 5–10 mm. above the top of the cathode.   Having assembled the

cell, the solution is heated to boiling and the desired E.M.F. is applied. Complete deposition usually requires from 10–25 minutes. Heating is then discontinued and, without breaking the circuit, the lower portion of the electrolysis vessel is immersed in a beaker of cold water. When the solution is cold, the anode and the cathode are successively withdrawn. The latter is rinsed successively with water, alcohol, and ether and is then dried as before, the tips of the platinum leading wires being ignited to remove traces of mercury. After cooling, the cathode is reweighed.

(a)   (b)
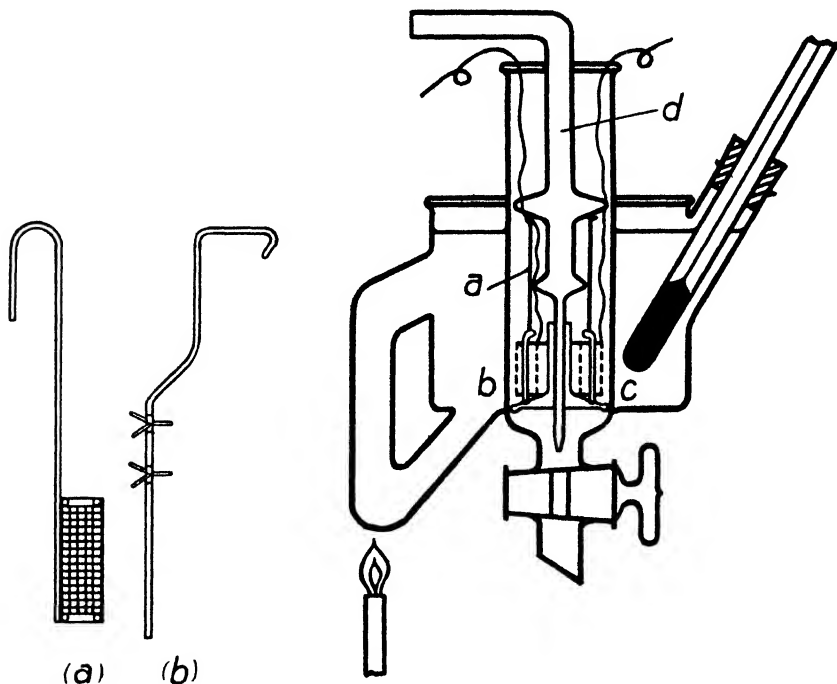
Fig. V.44.   Pregl Electrodes.   Fig. V.45.   The Clarke and Hermance Cell.

2. The Clarke and Hermance Cell.* Though more complicated than that of the Pregl cell, the design of the Clarke-Hermance cell (fig. V.45) permits effective agitation of the solution even when heating is not employed. Increased precision is thereby obtained.† The outer jacket is filled with water and may be heated as shown when necessary. The solution to be electrolysed (approximately 5 ml.) is contained in the inner portion. The stopcock enables the solution to be run off at the completion of the deposition, while water is added simultaneously at the top of the cell. Difficulties due to the re-solution of the deposit may thus be overcome.

* *J. Amer. Chem. Soc.*, 1932, **54**, 877.
† See, for example, MacNevin and Bournique, *Ind. Eng. Chem.* (Anal. Edn.), 1940, **12**, 431.

The electrode assembly is shown in figs. V.45 and V.46. Glass frame *a* allows the electrodes *b* and *c*, which are cylinders of platinum gauze, to be held concentrically within the cell. The cylinders are 15 mm. high and have inside diameters of 9 and 18 mm. respectively; a platinum leading wire is welded to each. Air tube *d* slips into the centre tube of the frame, so that the larger bulb rests upon the top of the frame. On introducing air or other gas through *d*, the stream of rising bubbles causes a continuous overflow of liquid from the top of the frame, so that the whole of the solution in the inter-electrode space is repeatedly changed. Simultaneous cathodic and anodic depositions may be carried out, since the electrodes are of approximately the same size. Both electrodes are then cleaned, dried, and
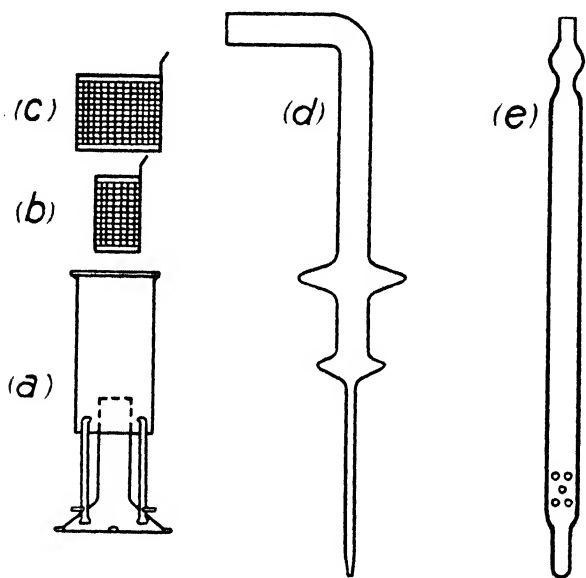
Fig. V.46.   Electrode Assembly of Clarke and Hermance Cell.

weighed as described in connection with the Pregl cell (p. 486). Usually, however, the inner electrode only is weighed and normally acts as the cathode.

Having prepared the electrodes, these are then mounted on the frame and the assembly is lowered into the cell containing the solution to be electrolysed. The level of the solution should coincide with the upper end of the centre tube of the frame. The air tube having been inserted, the air supply is adjusted until steady circulation of the solution is obtained. The desired E.M.F. is now applied to the electrodes. When the deposition is complete, as indicated by the current having become very small, the solution is drained from the cell whilst simultaneously adding water at the top of the

cell. Rinsing, drying, and reweighing are then carried out in the usual manner.

When the solution is required without excessive dilution for further examination and only the inner electrode has to be weighed, the washing tube shown at e in fig. V.46 is useful. At the end of the electrolysis the air tube is removed and the washing tube, the upper end of which is connected to a supply of distilled water, is inserted in its place. The cell is then slowly drained whilst admitting water at the same rate. Without interrupting the current, the inner electrode is raised until level with the perforations in the washing tube. The latter and the inner electrode are then withdrawn together, so that the latter is bathed in almost pure water when leaving the bulk of the liquid in the cell.

(a)   (b)
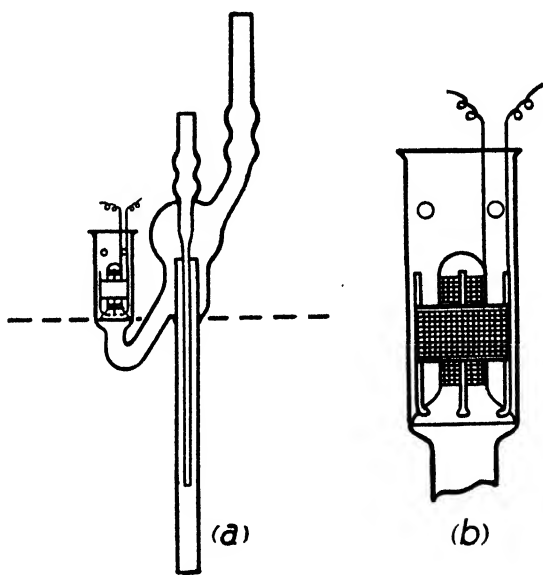
Fig. V.47.   Apparatus for Trace-metal Determination in Large Volumes of Solution.

By suitable modification, the cell may be employed to examine samples of solution as large as several hundred millilitres; a later development* is the combined air lift pump and electrolysis cell (fig. V.47, a and b). This may be used to determine traces of metal in very large volumes of solution (for example, 0·1 mg. of copper in 20 l. of solution). A stream of air bubbles rising from the jet lifts the solution and causes it to overflow from the top of the pump tube into the electrolysis chamber. The solution is diverted through the meshes of the electrodes by means of the solid glass ball surmounting the inner electrode, and finally flows back into the bulk of the liquid by way of the four holes near the top of the electrolysis chamber.

* Clarke and Hermance, *Mikrochem.*, 1936, **20**, 126.

3. The Lindsey and Sand Cell.* A "Monax" test-tube of 20 mm. internal diameter, shortened to an overall length of 100 mm., is used as the electrolysis vessel of this cell (fig. V.48), which was designed especially for micro-deposition under controlled potential. To cover the electrodes 12 ml. of solution are required. A current of air or other gas which is bubbled through the solution provides effective stirring, while a 100 ml. beaker, suitably heated by means of a micro-burner attached to the stand, serves as a water-bath.

To carry the electrodes, a small glass frame is sealed on near the lower end of the gas inlet tube, as shown at *a* in fig. V.49. A horizontal glass rod, which can be held in a boss-head on the stand, is sealed on at the upper end of the gas tube, and thus serves as a means of support; it also carries two small mercury cups for making electrical connections. The outer electrode, which normally acts as the anode, is of thin platinum gauze, as shown at *b*. It is held in position by the glass beads sealed to the exterior of the frame. The leading wire, the end of which dips into one of the mercury cups, is twisted round two beads sealed on the upper portion of the gas inlet tube. The inner electrode *c* is the ordinary Pregl cathode with the beads removed. This drops into the frame, the leading wire dipping into the second mercury cup.
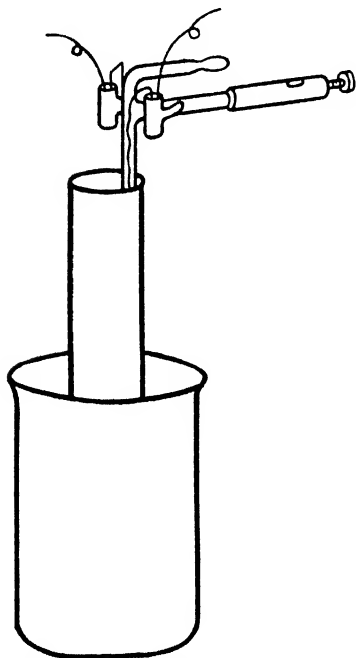
Fig. V.48.   The Lindsey and Sand Cell.

Having prepared the electrodes, they are assembled upon the frame, which is supported by the boss-head on the stand. The solution to be examined is placed in the electrolysis vessel, which is raised until the air tube just touches the bottom; if the electrodes are not completely immersed, a little water is added. The support carrying the water-bath and micro-burner is then raised into position. Having started and adjusted the air stream, the bath is heated to the desired temperature, when the electrolysis is carried out in the usual manner. When the current has fallen to about one-tenth of its initial value, the walls of the vessel are rinsed down with a fine jet of water and the electrolysis is continued for a further 2–3 minutes. Without interrupting either the current or the gas stream, the water-bath is replaced by a beaker containing cold water. When the solution has cooled to room temperature, the beaker is removed and the electrolysis

* *Analyst*, 1935, **60**, 739.

vessel is lowered, while the cathode is thoroughly washed by a fine jet of water. The cathode is then rinsed and dried, traces of mercury being driven from the tip of the leading wire by ignition. It is then cooled and reweighed.
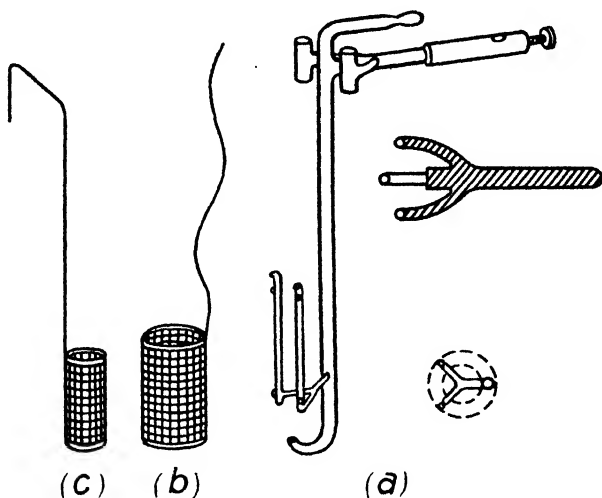
Fig. V.49. Electrode Assembly of Lindsey and Sand Cell.

In connection with the manipulation of this apparatus, two pieces of apparatus were designed by Lindsey. These are the silver-plated electrode-carrier (fig. V.50) for transporting the electrode without handling, and the drying apparatus. The latter is shown in fig. V.51. The bath contains
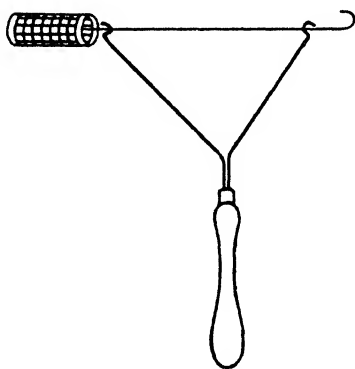
Fig. V.50. Lindsey Electrode-carrier.

a mixture of water and glycerin, so that temperatures in excess of 100° C. may be obtained. The electrode is hung from the lip of the drying chamber, while a stream of air, obtained by operating the hand-bellows and dried

by passage through calcium chloride, is blown in. In this apparatus most deposits can be dried to constant weight in 1 minute. When not in use, the drying chamber may be conveniently closed by means of a crucible lid.

REPRESENTATIVE EXAMPLES OF MICRO-ELECTRO-DEPOSITION

Example 1: Determination of Copper

GENERAL PRINCIPLES. Since copper may be deposited successfully from a variety of solutions, the electro-gravimetric method is particularly suited to the determination of this metal. In general, solutions containing free
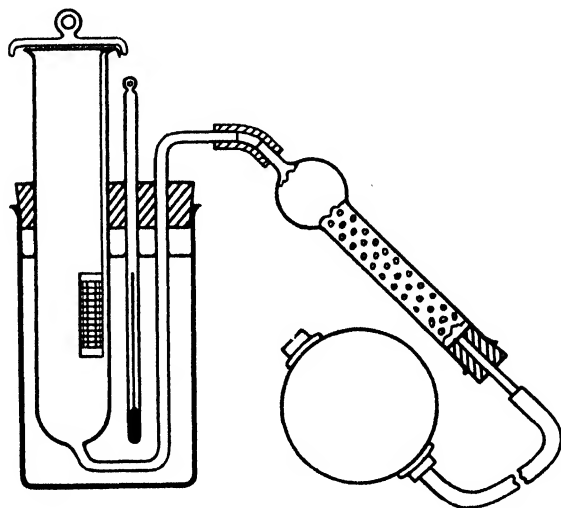
Fig. V.51. Drying Apparatus.

acid are employed, so that deposition of cuprous oxide does not occur. Evolution of hydrogen, which had a tendency to occur towards the end of the process, should be prevented by keeping the cathode potential constant; otherwise the deposit may be powdery. For the same reason an oxidising agent such as nitric acid should be added to the solution.

Although having little action alone, dilute nitric acid which contains oxides of nitrogen rapidly attacks the copper deposit. On the micro scale passage through the solution of a stream of air during the electrolysis removes these oxides continuously. Final traces can be destroyed by addition of urea or a similar reagent.

To avoid high results, filtration of the solution is essential. Unless removed, suspended silica and graphite tend to co-precipitate with the copper.

The addition of a few drops of alcohol to the solution helps to eliminate spray formation.

*Application to the Determination of Copper in Copper-Iron Alloys**

On the macro scale, quantitative deposition of copper from solutions containing appreciable quantities of iron is impractical, unless special techniques such as that of internal electrolysis, which allow the iron to be maintained in the ferrous state, are employed. When the determination is carried out on the micro scale, however, most of the difficulties disappear and the presence of large amounts of iron may be tolerated. Quantities of from 0·3–2 mg. of copper may be determined directly with a precision of about 0·01 mg. Molybdenum, if present, deposits along with the copper as the black sesquioxide; its interference may be prevented by addition of phosphoric acid, or of ammonium bifluoride, and additional nitric acid. Chromium, nickel, and manganese may be present without causing interference.

METHOD. A slightly modified Hermance-Clarke cell (p. 487) is recommended. The capacity is increased to about 12 ml., the water-jacket being omitted. If samples containing less than about 1% of copper are to be examined, a funnel-shaped extension should be sealed on to the top of the cell so that 50 ml. of solution may be accommodated.

Before use the cathode is cleaned in hot dilute (2N) nitric acid, washed with water and alcohol, dried at 110° C. for 5 minutes, and weighed after cooling for 5 minutes.

Nitric acid is usually suitable for dissolving the sample, and should always be present in the solution. Sulphuric and perchloric acids may also be used, since their presence causes no interference.

For the electrolysis 5 ml. of solution, which should contain from 0·3–2 mg. of copper and from 0·3–0·5 ml. of 70% nitric acid, are required. If molybdenum is present, 0·15 ml. of 85% phosphoric acid is added. The solution is filtered through a sintered-glass micro-funnel into the electrolytic cell, rinsing in with several small quantities of water so that the final volume is from 9–10 ml. After adding 3 drops of alcohol, the air stream is adjusted to 2–3 bubbles per second and the solution is electrolysed for 20 minutes at 2·5–2·8 volts. Having added 20–30 mg. of urea, the cell walls are then rinsed down and the electrolysis is continued for another 5 minutes. The cell is then slowly drained, water being added simultaneously until the current falls to zero. During this operation the voltage must not be allowed to rise above 3·5. The cathode is then withdrawn, rinsed with alcohol and dried at 110° C., and reweighed after cooling for 5 minutes.

When the sample is of low copper content (0·2–1%) correspondingly larger weights of alloy (up to 200 mg.) have to be taken. In order to avoid

* MacNevin and Bournique, *Ind. Eng. Chem.* (Anal. Edn.), 1943, **15,** 759.

too high a concentration of other metals, the solution is diluted to about 50 ml. and is electrolysed in the cell fitted with an extension.

Example 2: Determination of Mercury

GENERAL PRINCIPLES. Mercury may be deposited from a variety of solutions, such as those containing nitric or hydrochloric acid, ammonia, alkali cyanide or sulphide, etc., and this is undoubtedly the best micro method of separating mercury quantitatively from other elements. However, most of the micro methods so far developed employ solutions containing nitric acid.

Although electrolytically deposited mercury has some tendency to alloy with platinum, the deposit is not very adherent, and a *gold* surface should be used. Unless a cathode of that element can be employed, the more usual platinum gauze should be gold plated before use.

The volatility of mercury is one source of difficulty in a gravimetric analysis. Special care is needed in drying the cathode after deposition* and weighing should follow as rapidly as possible. A final colorimetric estimation (p. 300) is recommended in trace analysis.

Another source of error is the co-deposition of traces of copper.

Application to the Determination of Mercury in Organic Substances†

The organic material is destroyed (pp. 90–91) and the mercury is brought into solution by heating with nitric acid in a sealed tube. After suitable dilution, the solution is electrolysed at 40° C., mercury being deposited and weighed upon a gold-plated cathode. Using about 5 mg. of material, the average error is $\pm 0.2\%$.

METHOD. Before use, the cathode of the Pregl apparatus (p. 485) is gold plated. Having dissolved 50 mg. of gold in aqua regia, the excess acid is removed by several evaporations to dryness. The residue is then dissolved in a solution of 0·65 g. of potassium cyanide in 5 ml. of water. The solution is transferred to the cell and the gold is deposited at 50° C., using an applied E.M.F. of 3·5 volts for 2 hours. The cathode is then washed, dried, and weighed as described below.

From 3–8 mg. of the substance to be examined are weighed out and decomposed by heating with 10 drops of concentrated nitric acid in a bomb-tube at 270°–280° C. for 2 hours (see p. 91). When cold, the tube is opened and the contents are washed into the cell, so that the final volume of liquid is approximately 5 ml. Electrolysis is carried out at 40° C., in a water-bath, using an applied E.M.F. of 3·5 volts; the time required is 40 minutes. The solution is then cooled for 5 minutes, when the cathode is withdrawn and washed successively with water, alcohol, and ether. Since heating cannot

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* Hernler and Pfeningberger, *Mikrochem.*, 1936, **21**, 116.
† Verdino, *Mikrochem.*, 1928, **6**, 5.

be employed, drying is performed by waving the cathode in the air. The tip of the platinum leading wire is ignited and the cathode is then reweighed after an interval of 5 minutes.

To remove the deposit, the cathode should be gently heated over a small flame, taking precautions appropriate to the high toxicity of mercury vapour.

Example 3: Determinations of Bismuth and of Lead

Not only does *bismuth* tend to deposit in a spongy form, but pentoxide-formation on the anode may also occur. Efficient stirring, control of the cathode potential, and the presence of nitric acid enable the first difficulty to be overcome, while the presence of reducing agents such as hydroxylamine or hydrazine prevents anodic oxide deposition. Except in the presence of oxalic acid* coherent deposits are not obtained from chloride-containing solutions, owing to the formation of basic salts.

Though *lead* may be deposited cathodically in the metallic state, care must be taken to avoid simultaneous anodic deposition as the dioxide. Further, the ease with which the deposit is oxidised renders necessary rapid washing and drying, while preliminary plating of the cathode (for example, with silver or copper) is desirable to prevent the deposit alloying with the platinum.

Anodic deposition as lead dioxide from nearly boiling solution containing nitric acid is preferable, a high current density being used to expel the otherwise tenaciously held water from the deposit. Slightly high results are obtained, so that in calculating the weight of lead a factor lower than the theoretical 0·866 is employed.

When the two metals are present in the same solution, contamination of the anodic deposit by bismuth prevents lead from being separated first in the form of lead dioxide. Bismuth may, however, be deposited first if a reducing agent is added to the solution and the cathode potential is controlled. Deposition of lead may then be carried out either cathodically or, after destruction of the reducing agent, anodically.

Application to the Separation and Micro-determination of Bismuth and Lead†

PRINCIPLES. Bismuth is deposited with controlled cathode potential from nitric acid solution containing hydrazine hydrate. (This agent has the advantage of being easily destroyed, thus facilitating the subsequent determination of lead.‡) After destroying the latter, lead is deposited anodically as the dioxide. To avoid excessively high results due to occlusion of salts by the deposit, the latter is redissolved cathodically in nitric acid solution and is redeposited, a high current density being used for the reason stated above.

* Kny-Jones, *Analyst*, 1939, **64**, 172.
† Lindsey, *Analyst*, 1935, **60**, 744.
‡ Collin, *Analyst*, 1929, **54**, 654.

REAGENTS.

1. 50% *hydrazine hydrate solution.*
2. 50% *sodium hydroxide solution.*
3. *Sodium peroxide.*

METHOD. The Lindsey and Sand cell (see p. 490) is used in this analysis.

(*a*) *Determination of Bismuth.* The solution to be analysed, which should contain not more than 6 mg. of each metal, is introduced into the cell. Having added 1 ml. of nitric acid (sp. gr. 1·42) and 2 drops of hydrazine hydrate solution, the volume is brought up to 12–13 ml. by addition of water. The electrolysis is carried out at 60°–70° C. with nitrogen stirring, the inner electrode acting as the cathode and being maintained at 0·8 volt with respect to the anode. The current, initially about 70 milliamperes, decreases during the electrolysis to about 10 milliamperes.

After 10 minutes the walls of the cell are washed down with a fine jet of water; the voltage is then increased to 0·9 for a further 2 minutes. The cell is then cooled by replacing the water-bath by a beaker of cold water; with the current still on, the electrolysis vessel is replaced by a shorter one containing 12–14 ml. of water, the latter being retained for procedure (*b*). Having removed the cathode, it is dipped successively into alcohol and ether dried for 1 minute in the drying apparatus (p. 491); traces of mercury are then removed and the electrode is reweighed (p. 486).

(*b*) *Determination of Lead.* The solution, now free from bismuth, is transferred to a 50 ml. tall beaker, using portions of wash water from (*a*) to complete the transference. Sodium hydroxide solution is added dropwise until the precipitated lead hydroxide has just redissolved, when 10–20 mg. of sodium peroxide are added. The beaker is covered and heated until oxygen evolution is complete, when nitric acid (sp. gr. 1·42) is added until neutral, followed by 4 ml. in excess. The remainder of the wash water from (*a*) is then added, when the final volume should be from 30–35 ml.

Electrolysis is carried out, with nitrogen stirring, in the beaker, which is heated directly by a micro-burner so that the solution is just below boiling-point. The inner electrode acts as the *anode* and is maintained at about 1·2 volts with respect to the cathode (a higher voltage may be used and the current may be as great as 300 milliamperes). The deposition is complete in 15 minutes, the walls of the beaker having been washed down after 12 minutes.

Having replaced the beaker by an electrolysis vessel containing 2 ml. of nitric acid and 12 ml. of water, the current is reversed until deposit is no longer visible upon the inner electrode. The flow of current is then restored to its original direction and the lead dioxide is redeposited from an almost-boiling solution. The anode-to-cathode potential should be 1·0 volt, or higher, so that the current flowing is from 120 to 200 milliamperes. After 7 minutes the walls of the cell are washed down; in another 3 minutes

the electrolysis vessel is replaced by a shorter one containing water. The inner electrode is then removed, rinsed, dried, and reweighed as described above.

To calculate the weight of lead from that of the deposit the factor 0·860 is used.

TABLE IV

FURTHER EXAMPLES OF MICRO-ANALYSIS BY ELECTRO-DEPOSITION

Analysis	References
Arsenic.	Torrance, S., *Analyst*, 1939, **64**, 263.
Cadmium.	Fife, J. G., *Analyst*, 1938, **63**, 650.
Cobalt.	Okáč, A., *Z. Anal. Chem.*, 1932, **88**, 189; *Mikrochem.*, 1932, **12**, 205.
Copper.	Lindsey, A. J., *Analyst*, 1938, **63**, 159. Lecoq, H., *Bull. Soc. Roy. Sci. Liège*, 1942, **11**, 418, 614. Hernler, F., and Pfeningberger, R., *Mikrochem.*, 1936, **21**, 116. Llacer, A. J., Sozzi, J. A., and Benedetti-Pichler, A. A., *Ind. Eng. Chem.* (Anal. Edn.), 1941, **13**, 507. Wenger, P., Cimerman, C., and Tschanun, G., *Mikrochim. Acta.*, 1937, **1**, 51. Wiesenberger, E., *Mikrochem.*, 1931, **10**, 10.
Gold.	Hernler, F., and Pfeningberger, R., *Mikrochem.*, 1936, **21**, 116. Raeder, M. G., and Kyllingstad, O. S., *Mikrochem. ver. Mikrochim. Acta.*, 1939, **27**, 112. Fuchs, K., *Mikrochem.*, 1923, **1**, 86.
Lead.	Bambach, K., and Cholak, J., *Ind. Eng. Chem.* (Anal. Edn.), 1941, **13**, 504. Brantner, H., and Hecht, F., *Mikrochem.*, 1933, **14**, 27, 30. Fields, M., *New Zealand J. Sci. Tech.*, 1942, **23B**, 224. Lecoq, H., *Bull. Soc. Roy. Sci. Liège*, 1942, **11**, 418, 614. Muller, H., *Z. Anal. Chem.*, 1938, **113**, 161. Randall, M., and Sarquis, M. N., *Ind. Eng. Chem.* (Anal. Edn.), 1935, **7**, 2.
Mercury.	Hernler, F., and Pfeningberger, R., *Mikrochem.*, 1936, **21**, 116. Fife, J. G., *Analyst*, 1938, **63**, 650.
Nickel.	Fife, J. G., *Analyst*, 1936, **61**, 681. Okáč, A., *Z. Anal. Chem.*, 1932, **88**, 189. Llacer, A. J., Sozzi, J. A., and Benedetti-Pichler, A. A., *Ind. Eng. Chem.* (Anal. Edn.), 1941, 13, 507.
Rhenium.	Voigt, A., *Z. Anorg. allgem. Chem.*, 1942, **249**, 225.
Silver.	Friedrich, A., and Rapoport, S., *Mikrochem.*, 1935, **18**, 227.
Zinc.	Fife, J. G., *Analyst*, 1936, **61**, 681. Wenger, P., Cimerman, C., and Tschanun, G., *Mikrochim. Acta.*, 1937, **1**, 51.

CONDUCTOMETRIC TITRATION

General Principles

SINCE the conductivity of a solution depends upon the total number of ions present, removal of ions by precipitation (or complex-formation) causes the conductivity to decrease. Thus in the titration of a solution of magnesium sulphate with barium hydroxide solution, the conductivity falls progressively as reaction

$$Mg^{++} \quad SO_4^{--} + Ba^{++} \quad 2OH^- = BaSO_4 \downarrow + Mg(OH)_2 \downarrow$$
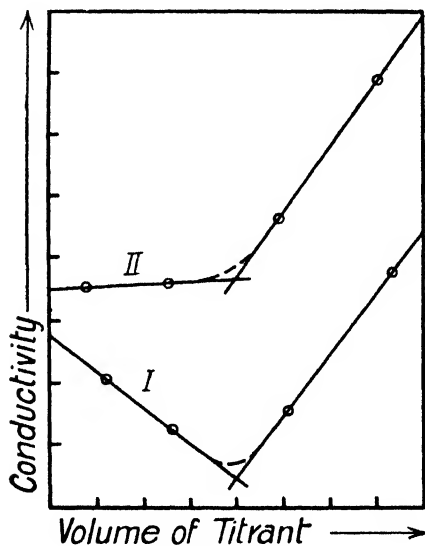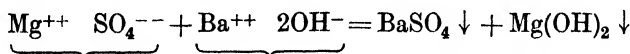
Fig. V.52.
Typical Conductometric Titration Curves.

proceeds. Continued addition of barium hydroxide beyond the end-point causes the ionic concentration, and hence the conductivity, to increase progressively. If the conductivity of the solution is plotted against the volume of reagent added a curve of type *I*, fig. V.52, is obtained. From this the end-point volume may be read off. The dilution of the test solution by introduction of the reagent may be corrected for as described on p. 474. Solubility of a precipitate causes the titration curve to be rounded at the end-point, but unless this is excessive no difficulty is experienced in locating the end-point graphically.

Another major factor influencing the conductivity of solutions is the mobility of the constituent ions. *Exchange* of one ion for another may thus be followed by noting a conductivity change in the electrolyte. This may be illustrated by the titration of a strong acid by a strong base, as for example:

$$H^+ \quad Cl^- + Na^+ \quad OH^- = Na^+ \quad Cl^- + H_2O$$
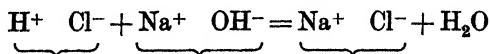
The highly-mobile hydrogen ions are progressively removed to form almost un-ionised water, and are replaced by the much less mobile sodium ions. Until the end-point has been reached the conductivity therefore

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falls. Continued addition of reagent then increases the concentration of hydroxyl ions, which are also very mobile, so that the conductivity now increases. A titration curve similar to that of curve I of fig. V.52 is thus obtained.

When the nature of the reaction is such that the ions removed from the solution are replaced by ones of similar mobility, little change in conductivity occurs. Thus in the titration of the salt of a weak acid by a strong acid ("displacement" reaction), as for example:

$$\underbrace{CH_3COO^- \quad Na^+} + \underbrace{H^+ \quad Cl^-} = \underbrace{Na^+ \quad Cl^-} + CH_3COOH$$
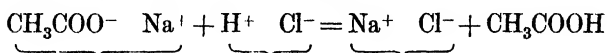
the liberated weak acid is but slightly ionised and contributes little to the

conductivity; in effect, the acetate ion is replaced by the somewhat more mobile chloride ion. The conductivity therefore shows but slight increase until the end-point is reached; continued addition of reagent then introduces highly mobile hydrogen ions, with consequent rapid increase in conductivity, as shown by Curve II, fig. V.52.

Analogous phenomena usually occur in precipitation reactions. For example, in the titration of a soluble chloride with silver nitrate solution:

$$\underbrace{Na^+ \quad Cl^-} + \underbrace{Ag^+ \quad NO_3^-} =$$

$$\underbrace{Na^+ \quad NO_3^-} + AgCl \downarrow$$

Fig. V.53. Titration of a Mixture of a Strong and a Weak Acid.

Up to the end-point, the net effect is the replacement of chloride ions by nitrate ions of similar mobility; continued addition of reagent then increases the total ionic concentration and hence the conductivity. For an example, see p. 501.

Depending upon the reacting substances and their concentrations, a variety of other forms of titration curve may be obtained.* In certain cases, as, for example, in the titration of a mixture of a weak and a strong acid by a strong base, two substances present in the same solution may be determined by a single titration. The titration curve (fig. V.53) has two breaks, corresponding to the respective neutralisation points of the strong and of the weak acid.

* For a discussion, see Britton, H. T. S., "Conductometric Analysis" (London, 1934).

APPARATUS AND GENERAL TECHNIQUE

The application of conductometric methods to micro-titration has been due almost entirely to Jander and his co-workers.* They showed that quantities of a few micrograms of various ions could be titrated with considerable precision.

1. Titration Assembly. The cell, which may have a capacity of from 5–50 ml., is shown in fig. V.54. For the examination of poorly conducting solutions the platinum electrodes may be arranged in the form of concentric half-circles spaced 2 mm. apart, as shown at *a*. Resistance glass should be employed in construction, and the cells should be well steamed out before use.

Except for use with extremely dilute solutions (when the absorptive effect introduces errors), the electrodes are coated with platinum black by covering them with 3% platinic chloride and passing a current. The latter may be drawn from a 4 volt accumulator, a reversing switch being incorporated so that the current through the cell may be reversed every half-minute or so until both electrodes have an even, black deposit. Traces of chlorine are then removed by continuing the electrolysis in dilute sulphuric acid. Thorough washing completes the preparation; when not in use, the deposit should be prevented from drying out by filling the cell with water.
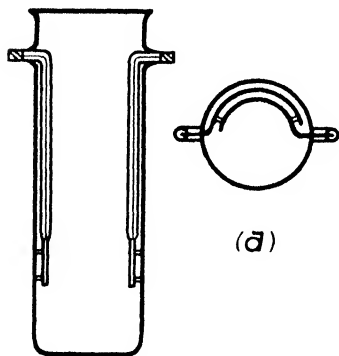
During titration the solution is stirred mechanically and, when necessary, a current of nitrogen is passed over its surface to exclude atmospheric carbon dioxide, etc. A mechanically operated micro-burette graduated in divisions of 0·0005 ml. was developed by Jander and Harms; others of suitable capacity (see p. 154) may be used.

(*a*)

Fig. V.54.   Titration Cell.

Though graphical end-point location permits rapid working, the high temperature coefficient of electrolytic conductivity necessitates avoidance of temperature-changes during titration. In addition to normal precautions, the measuring current, and hence the internal heating effect, should be small.

2. Measurement of Conductivity. Since the sole aim is to detect the titration end-point, it is unnecessary to measure the absolute conductivity of the solution. Measurement of relative conductivity, or of a quantity related to it, is sufficient.

Alternating current is usually employed, the aim being that the polarising

* Jander and Harms, *Angew. Chem.*, 1935, **48**, 267; *Z. Elektrochem.*, 1936, **42**, 315; Jander and Immig, ibid., 1937, **43**, 207, 211, 214.

effects of the equal and opposite pulses of current shall neutralise one another. A simple method of generating the required alternating current is by means of a small induction coil, which is, however, mechanically noisy and yields unsymmetrical pulses. A thermionic valve oscillator* is preferable, since it is free from these drawbacks and possesses the additional advantage that the frequency of the pulsations is readily controllable. Another form of generator is a motor-driven alternator; while low-frequency pulsations may be obtained from a suitable transformer connected to the A.C. mains.

For measurement, the usual Wheatstone bridge arrangement may be used, the null point being detected by a telephone earpiece, an A.C. galvanometer, or indicator employing thermionic valves. While the first is simplest, very quiet laboratory conditions are required. The valve indicator may be incorporated with the bridge network into a compact unit.†

Alternatively, a continuous-reading method may be used, so that manipulation of electrical instruments during titration is eliminated. A constant alternating voltage is applied to the electrodes and the current strength, instead of the conductivity of the solution, is plotted against the volume of reagent added.‡ To measure the current, an A.C. galvanometer or, in conjunction with a commutator or other rectifying device, a D.C. galvanometer may be used.§ A preferable arrangement is the use of a thermionic valve system. A compact assembly employing a robust D.C. milliammeter or microammeter is thus possible. An oscillator circuit may also be incorporated, so that a single unit thus serves for the generation of alternating current and for the continuous reading of the current flowing through the cell.||

Example: The Determination of Soluble Chloride

The titration of chloride with silver nitrate solution¶ (see p. 499) was applied to the micro scale by Jander and Immig.** Using a 40 ml. cell (fig. V.54, p. 500) these workers showed that quantities of 0·2 mg. and upwards of chloride (expressed as chlorine) may be titrated with an average error of less than 0·5%. Electrodes are coated with platinum black only when the amount of chloride exceeds 1 mg.

By working with a volume of from 3–4 ml. of solution in a small cell with electrodes arranged as shown at a in fig. V.54 (p. 500), the lower limit for

* See, for example, Woolcock and Murray-Rust, *Phil. Mag.*, 1928, VII, **5**, 1130; Jones and Josephs, *J. Amer. Chem. Soc.*, 1928, **50**, 1049.

† Mullard Wireless Service Co., Ltd., London; "Mullard Measuring Bridge, Type G.M. 4140."

‡ Treadwell and Janett, *Helv. Chim. Acta*, 1923, **6**, 734.

§ See, for example, Jander and Pfundt, *Z. Elektrochem.*, 1929, **35**, 206; Jander and Schornstein, *Z. angew. Chem.*, 1932, **45**, 701.

|| See, for example, Ehrhardt, *Chem. Fabrik*, 1929, **2**, 443, 455, 463; Garman, *Ind. Eng. Chem.* (Anal. Edn.), 1936, **8**, 146.

¶ Kolthoff, *Z. anal. Chem.*, 1923, **64**, 229.

** Jander and Immig, *Z. Elektrochem.*, 1937, **43**, 211.

accurate titration may be correspondingly reduced. As the amount of chloride present is decreased, however, the effect of the solubility of silver chloride and of traces of impurities becomes proportionately greater, so that in aqueous solution quantities of chloride less than 10 μg. cannot be titrated.

The use of an alcoholic medium reduces the solubility of silver chloride (compare p. 480) and thus allows even smaller amounts of chloride to be determined. Very careful purification of the alcohol is necessary, while entry of atmospheric oxygen or carbon dioxide during the titration must be prevented. In this manner, quantities of chloride of about 1 μg. may be titrated with an error of about 10%.

Application to the Determination of Chloride in Tap-Water

Tap-water normally contains about 10–20 mg. per litre of chloride, so that a sample no larger than a few millilitres is sufficient if titrated conductometrically.

REAGENTS.

1. *Silver nitrate solution*, 0·0100N. 1 ml. ≡ 0·355 mg. of chloride.

2. *Double distilled water*.

METHOD. 3 ml. of tap-water are pipetted into the cell, which should be of 10 ml. capacity, and 7 ml. of double distilled water are added. Having completed the electrical connections, the stirrer is started and the micro-burette, which should have a capacity of about 0·5 ml., is introduced. The reagent is added in quantities of from 0·01–0·02 ml., noting the relative conductivity after each addition. To permit the construction of a titration curve, from which the end-point volume of reagent may be read off, the titration is continued well past the point of minimum conductivity. Owing to the small volume of reagent added, correction for its diluting effect (see p. 474) is unnecessary.

TABLE V

FURTHER EXAMPLES OF MICRO-CONDUCTOMETRIC TITRATION

Analysis	References
Silver (in presence of lead).	Jander, G., and Immig, H., Z. Elektrochem., 1937, **43**, 214,
Silver, copper, cadmium, lead, and bismuth (with hydrogen sulphide).	Jander, G., and Immig, H., Z. Elektrochem., 1937, **43**, 207.
Arsenic (with iodine).	Jander, G., and Harms, J., Angew. Chem., 1935, **48**, 267.
Phosphates.	Chretien, A., and Kraft, J., Bull. Soc. chim., 1938, **5** (5), 1399.
Fluoride (with aluminium chloride).	Jander, G., and Harms, J., Z. Elektrochem., 1936, **42**, 315.
Iodate (with hydrochloric acid).	Murgulescu, I. G., and Latiu, E., Z. Anal. Chem., 1943, **125**, 265.
Selenite (with lead nitrate).	Ripan-Tilici, R., Z. Anal. Chem., 1938, **114**, 409.
Sulphur (in organic substances).	Scholz, G., Z. Anal. Chem., 1944, **127**, 11.
Nicotine (in tobacco distillates).	Ripan-Tilici, R., and Cristea, F., Z. Unters. Lebensm., 1938, **76**, 44; 1939, **77**, 283.

PART VI

GASOMETRIC METHODS OF MICRO-ANALYSIS

By K. M. Wilson

                                APPENDIX

PART VI

GASOMETRIC METHODS OF MICRO-ANALYSIS

GENERAL INTRODUCTION

MICRO-CHEMICAL methods involving the measurement of gases have been in use from the early years of the present century, and special techniques have been developed to meet the varying needs of research workers in fields of agriculture, biology, glass technology, and lamp manufacture, to mention but a few. As would be expected, the procedures which have been developed follow different lines, and many of these, as, for example, low-temperature condensation methods* for the analysis of minute amounts of gas in low-pressure systems and micro-respirometer techniques,† used for the study of the respiration of tiny fragments of living tissue, are extremely specialised. There are, however, a number of gasometric techniques capable of wide application in the field of analytical chemistry. From these, a sufficiently representative selection has been made to indicate their potentialities, bearing in mind the practical need for simple apparatus and readily available reagents. In this way it has been possible to present an adequate description of each form of apparatus and of the corresponding experimental procedure. To assist readers who may wish to obtain more information than is given in the following pages, the most important key references have been given in each section. Useful surveys of this subject have, however, been made by Farkas and Melville,‡ Ambler,§ and others.||

The classical methods of Hempel, using 100 ml. samples, depend on the successive absorption of the various constituents of any gas and measurement of the residues under conditions of constant temperature and pressure. They require about 200 ml. of each reagent. Orsat's development of this apparatus is widely used and portable, though still bulky.

The well-known Haldane apparatus, designed at the beginning of the century for the analysis of mine atmospheres, uses a 10 ml. sample, and by introducing thermo-barometric compensation, achieved greater accuracy. Many modifications, some of a truly micro nature,¶ exist and find their

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* Langmuir, *J.A.C.S.*, 1912, **34**, 1310; Ryder, ibid., 1918, **40**, 1656.

† Heatley, Berenblum, and Chain, *Biochem. J.*, 1939, **33**, 53; Boell, Needham, and Rogers, *Proc. Roy. Soc.*, 1939, **B. 127**, 322.

‡ Farkas and Melville, "Experimental Methods in Gas Reactions" (Macmillan, London, 1939).

§ Bunge and Ambler, "Technical Gas Analysis" (Gurney and Jackson, London, 1934).

|| Hartridge, *J. Sci. Instr.*, 1939, **16**, 317.

¶ Burk and Milner, *Ind. Eng. Chem.* (Anal. Edn.), 1932, **4**, 3; Perkins, ibid., 1943, **15**, 61.

application mainly in the biological field. More recently, Ambler's apparatus* has come into favour for industrial gas analysis. As with the Haldane apparatus, a 10 ml. sample is required, but the method is much more convenient for combustible gases and the arrangement permits of great economy of reagents.

Two truly micro-chemical techniques, which require quantities of gas of the order of only 10–50 cu. mm., are described on pp. 509–522.

An alternative principle, viz. the measurement of pressure changes in a sample maintained at constant volume, is used in the apparatus devised by Bone and Wheeler for samples of the order of 10 ml. and in recent designs an accuracy of $\pm 0.02\%$ can be reached. An application of this principle on the micro-chemical scale is described on pp. 523–529.

In addition, micro gas analysis by measurement, under a microscope, of the diameter of a gas bubble (see pp. 530–533) makes use of quite a novel principle which is not applicable on the larger scale.

In a micro-analytical laboratory gasometric techniques, however, have a second and possibly far more important aspect than the mere analysis of mixed gases. Physiologists and biochemists, notably Krogh, Barcroft, Van Slyke, and Warburg, have shown that much of the scientific data required in physiological research, clinical medicine, and applied biology can be obtained most simply by measuring gases evolved in chemical or biological reactions of quite small (milligram scale) masses of organic material.

Van Slyke's methods involve essentially the measurement of gases such as oxygen, carbon dioxide, carbon monoxide, or nitrogen which may be evolved quantitatively from organic substances in the course of chemical reactions. Of these, only the more accurate constant-volume technique is described in this volume.

Other manometric methods, developed primarily for the study of cell respiration, etc., can often be applied to the problems of analytical chemistry. Amongst these, Warburg's apparatus, comprising a flask and a simple manometer, is the most adaptable, though for some purposes the slightly more complex differential manometer of Barcroft has advantages. They are particularly useful for following rates of change in micro-chemical processes. Furthermore, reactions in which hydrogen ions are produced can be followed by measurement of carbon dioxide evolution from a bicarbonate buffer. Since these applications of manometric measurements have been dealt with comprehensively in M. Dixon's monograph on this subject† they need not be summarised in this book, which instances only a few manometric analyses of some technical value.

Consideration of the following pages will show that gasometric techniques, though usually devised for specialised purposes, make use of general principles, and that very often a particular apparatus can be used for many

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* Bunge and Ambler, "Technical Gas Analysis" (Gurney and Jackson, London, 1934).
† Dixon, M., "Manometric Methods" (2nd edn., Cambridge University Press, 1943).

analyses of different types. It should not be forgotten, however, that each type of apparatus has its own manipulative snags which must be appreciated before novel applications are considered, and consequently it is important to study in detail the typical procedures which are described in each section and to realise their limitations as well as their advantages.

THE MICRO-ANALYSIS OF GASES

A. CONSTANT PRESSURE METHODS

In recent years, a number of variable-volume capillary burette methods for the analysis of gases have been developed. They are based on the principles laid down by Reeve[1] and Christiansen,[2] and invariably use solid reagents and dry gas samples. Of these methods, those of Blacet and his colleagues,* and of T. C. Sutton† are the most promising. Whilst a high degree of precision can be achieved, there is still room for improvement, especially in devising truly specific reagents for single gases, or groups of gases.

(a) Blacet and Leighton's Technique

PRINCIPLES. The sample for analysis, *which must be quite dry*, is stored over mercury in miniature gas-holders, and from there is drawn into a capillary burette. The bubble of gas is adjusted to constant pressure and its initial volume is measured in arbitrary units of length. After this, the gas is expelled into another gas-holder and a platinum loop carrying a *solid* absorption reagent, or a liquid of low vapour pressure absorbed in a porous bead, is introduced through the mercury. After the absorption of each individual constituent, the gas is returned to the burette and its new length is measured.

"Hot wire," spark, and catalytic combustions may be effected by means of simple auxiliary apparatus.

The precision of analysis is about 0·1% for samples of the order of 50 cu. mm. volume.

APPARATUS (fig. VI.1, p. 511). The gas burette *a* is made from uniform capillary tubing of 0·5 mm. bore, graduated over some 45 cm. of its length and surrounded by a water-jacket *b*. It must be calibrated as accurately as possible, preferably by weighing mercury (see p. 155). The lower end of the capillary burette is enlarged and fitted with a device for advancing the mercury column by displacement from the reservoir *c*.

Reservoir *c* is constructed of steel and leakage is obviated by the rubber sleeve *d*, stretched over the loose steel plunger *e*. A fine-pitch screw advances the plunger and so stretches the rubber, which, however, remains clamped

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[1] *J. Chem. Soc.*, 1924, **125**, 1946.   [2] *J. Amer. Chem. Soc.*, 1925, **47**, 109.
* Blacet, F. E., and Leighton, P. A., *Ind. Eng. Chem.* (Anal. Edn.), 1931, **3**, 266; Blacet, F. E., Macdonald, G. D., and Leighton, P. A., ibid., 1933, **5**, 272; Blacet and Macdonald, ibid., 1934, **6**, 334; Blacet and Volman, D. H., ibid., 1937, **9**, 44; see also Swearingen, J. S., Gerbes, O., and Ellis, E. W., ibid., 1933, **5**, 369; Seevers, M. H., and Stormont, R. T., ibid., 1937, **9**, 39.   † Sutton, T. C., *J. Sci. Inst.*, 1938, **15**, 133.

by the gland and nut *f*. Both the water-jacket and capillary tube are sealed to the assembly by picein wax or a similar compound.*

The upper end of the burette terminates in a fine vertical nozzle with its tip rounded to a radius slightly less than that of the gas-holders *g*. It is important that this tip should reach the *extreme end* of the gas-holders, otherwise quantitative transfer of gas is impossible.

The large trough *T*, which can be raised or lowered by a screw, is filled with mercury, the level of which can be adjusted to a fixed ivory pointer *P*.
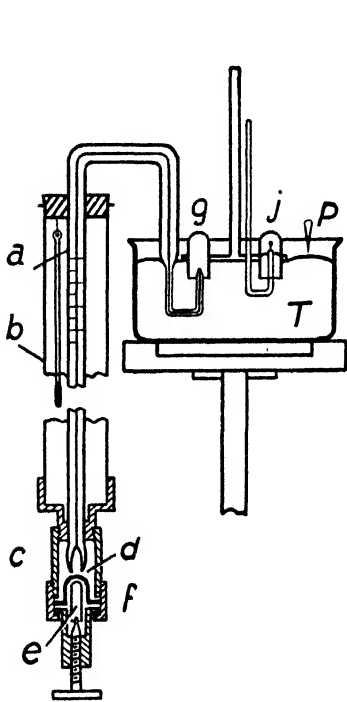
Fig. VI.1. Blacet and Leighton's Apparatus for Micro Gas Analysis.
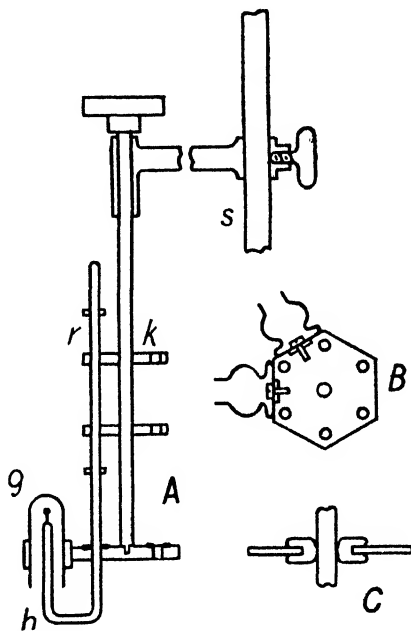
Fig. VI.2. Blacet and Leighton's Apparatus. *A*, rotating clamp for gas-holders and reagent carriers. *B*, details of clips, etc., on gas-holders. *C*, rubber grommet fitting for reagent-holders, *r*.

A standard *S* attached to the platform carries small gas-holders of about 2 ml. capacity in a rotating clamp *A* (fig. VI.2).

The reagent-holders *h* are short lengths of platinum wire fused into the ends of J-shaped glass rods *r* which slide between guide-plates *k* and are fitted with rubber limit stops. As with the capillary jet, the platinum wire must be capable of reaching the extreme end of the gas-holder.

When analyses involving "hot" reactions are to be undertaken the following accessories are required.

* Picein wax alone is rather brittle; a small quantity of Chatterton's compound mixed with it serves as a plasticiser and improves the adhesive qualities.

(1) *Combustion Wire.* About 1·5 cm. of 24 S.W.G. platinum wire is fused into the end of a 1 mm. bore soda-glass capillary tube of the same diameter and shape as the reagent-holder rods. Connection inside the capillary is made by means of a bead of mercury and copper wire. The projecting platinum wire is bent into an inverted **U**. When inserted into the gas-holder, a current can be passed through the platinum *via* the copper wire and the mercury in the trough.

(2) *Spark Electrodes.* A capillary tube of about 3 mm. outside diameter is fitted inside a quill tube of 4 mm. bore. Short lengths of platinum wire are placed (i) in the bore of the centre tube and (ii) in the annulus between the
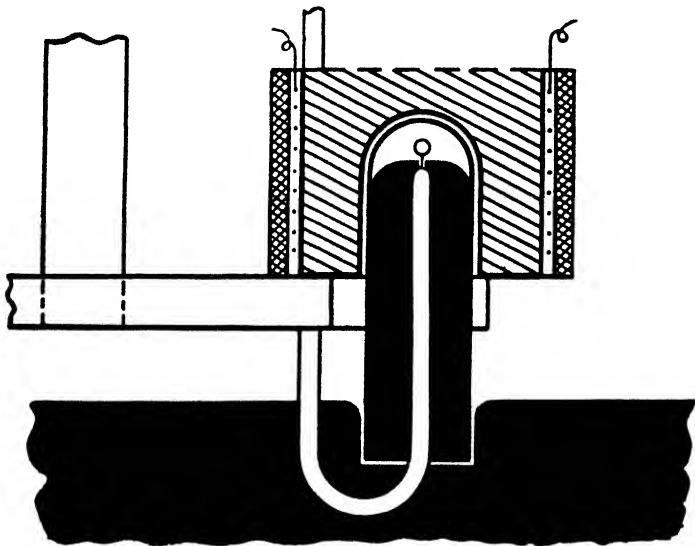
Fig. VI.3.   Blacet and Leighton's Apparatus: Heater for carrying out reactions at elevated temperatures.

centre and outer tubes, and the two glass tubes are fused together, to leave a hemispherical end with the two insulated wires separated by about 1 mm. The projecting wires are cut at the surface of the glass to leave a smooth round end, and connections inside the tubes are made to each with thin copper wire.

(3) *Heater for Catalytic Combustions* (fig. VI.3). The copper vessel, about 2·5 cm. deep and 1·5 cm. wide, wound on the outside with a suitably insulated resistance wire, has a central cavity which can fit snugly over the gas-holder, as shown. It is attached to an iron stand, which also carries a clip for the gas-holder. In use, the annular part of the vessel is filled with a stable substance, such as lead, which melts at the required temperature.

REAGENTS.

General Considerations. To avoid loss of gas by solution or complications due to vapour pressure effects, solid substances should, wherever possible, be used in analyses conducted with the capillary burette technique. Liquid reagents may be absorbed into a small porous bead, but then care must be taken to dry the remaining gas before it is drawn into the burette. Similarly, if the reagent gives off a vapour (e.g. SO_3 from fuming sulphuric acid), this must be removed prior to measurement.

The actual amount of reagent should be small—not larger than a 2 mm. sphere—and should be immersed under mercury as soon as it has been prepared so as to prevent inactivation of the surface. Further, reagents should always be handled with forceps, preferably of stainless steel, and *never* with the fingers. Fusion of reagents in a gas flame must be avoided at all costs since the products of combustion invariably spoil the absorbent. The most satisfactory method of fusion is to use a small resistance coil capable of reaching a bright red heat in free air. Alternatively, a copper block (fig. VI.4) may be heated in a gas flame and then placed over the reagent-holder to fuse the substance on the platinum wire. Nitrogen, or other suitable gas, may be led through the hollow handle to protect the reagent from oxidation, etc., during the fusion process.
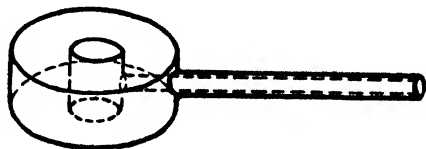
Fig. VI.4. Blacet and Leighton's Apparatus: Copper block heater for preparation of beads.

*Preparation of Porous Beads.** A platinum loop is dipped into a paste made from porous pot (sieved to 120–140 mesh) and kaolin in the proportions 70/30, and the resulting bead is baked in a clear Bunsen flame until coherent. To avoid trapping air these beads are charged with liquid by immersing them *slowly* below the surface of the reagent. Excess of liquid is then removed with filter-paper until the surface of the bead is *just* moist.

Fixative for Reagents Unstable on Fusion. Reagents such as silver oxide should be attached to *straight* wires by using the *minimum* amount of a concentrated sodium silicate solution and taking precautions to avoid covering the active surface with the silicate. After drying at room temperature in an inert atmosphere for 15 minutes, the reagent is ready for use.

Absorbents for Water Vapour

(a) *Fused Phosphorus Pentoxide.* Place a fragment of phosphorus pentoxide in a platinum loop and fuse it by means of a resistance coil or a hot copper block (fig. VI.4). Immerse the bead under mercury as soon as it is cold.

* Swearingen, J. S., Gerbes, O., and Ellis, E. W., *Ind. Eng. Chem.* (Anal. Edn.), 1933, **5**, 369.

Absorption is complete in 1–2 minutes.

The reagent will also absorb ammonia.

(b) *A Porous Bead Charged with Concentrated Sulphuric Acid.* For preparation see p. 513. The reagent also absorbs ammonia and olefines.

(c) *Fused Caustic Potash (Dry).* Prepare as described under (a) above. Absorption takes 5 minutes. Carbon dioxide is also absorbed.

Absorbents for Oxygen

(a) *Yellow Phosphorus.* Fuse a fragment of yellow phosphorus, freshly cut from the inside of a stick, on to a platinum loop, using the heated copper block and maintaining within it an atmosphere of nitrogen until the globule is just solid. Then plunge the bead into water, wash it several times in alcohol, and finally dry it in a stream of nitrogen. The washing clears the surface of the bead from phosphoric acid and makes it so active that spontaneous combustion may occur in gas samples of high oxygen content. Hydrocarbons may be partly absorbed.

(b) *Chromous Sulphate Solution.** This may be used on a porous bead.

Absorbents for Carbon Dioxide

Fused Caustic Potash (Moist). Expose a freshly made bead to the air until the surface just becomes shiny. Then keep under mercury.

Absorbents for Carbon Monoxide

(a) *Silver Oxide.* Precipitate the grey oxide from silver nitrate by the cautious addition of ammonia, wash very thoroughly by decantation, and press the moist mass into small pellets. Dry these at room temperature in a carbon dioxide free atmosphere, and attach pieces of about 1·5 mm. diameter to straight platinum wires by means of sodium silicate (p. 513).

Carbon monoxide is selectively oxidised and then completely absorbed by these pellets in 15 minutes. Hydrogen is very slightly oxidised too, but in 15 minutes the attack on hydrogen occurs to the extent of only 1%.

(b) *Copper Oxide/Caustic Potash (Heated).* This is the reagent also used for hydrogen removal (see below).

Absorbent for Olefines

Fuming Sulphuric Containing 30% of Sulphur Trioxide. This is used in a porous bead. After use it must be followed by a caustic potash bead for the purpose of removing sulphur trioxide vapour before the residual gas is measured.

*Stone, H. W., *J. Amer. Chem. Soc.*, 1936, **58**, 2591.

Absorbent for Acetylene

Alkaline Cuprous Chloride. Moisten cuprous chloride with dilute caustic potash, press the paste into a platinum loop, and dry carefully, taking care not to darken the surface of the bead by over-heating.

Absorption of acetylene is complete in under 5 minutes.

Absorbents for Hydrogen

(a) *Copper Oxide/Caustic Potash* (at temperature of molten lead). Fuse a little cupric oxide on to a platinum loop and, whilst still molten, add a fragment of *carbonate-free* caustic potash, avoiding excess. The two substances should coalesce to form a dark blue bead which, when heated in the apparatus described on p. 512 (fig. VI.3), with molten lead as the indicator of the temperature, is an active absorbent for hydrogen.

Carbon monoxide is also attacked.

(b) *Colloidal Palladium in a Porous Bead.* Compare p. 513.

Analytical Procedure. In any analysis of a gas mixture, it is essential to plan the operational procedure from a knowledge of the constituents which have to be removed.

The table on pp. 516–517, which summarises the various absorbents available and their reactions, may be used as a guide in deciding the sequence in which the various constituents are to be removed and the appropriate reagents to choose.

Thus for the complete analysis of a gas sample containing (i) water vapour, (ii) hydrogen, (iii) oxygen, (iv) nitrogen, (v) carbon dioxide, and (vi) ethylene it becomes evident that the order of analysis should be:

(a) *Absorption of water* with *phosphorus pentoxide.*

(b) *Absorption of carbon dioxide* with *caustic potash.*

(c) *Absorption of ethylene* with *fuming sulphuric acid,* following this with caustic potash again.

(d) *Absorption of oxygen* with *yellow phosphorus.*

(e) *Absorption of hydrogen* with *copper oxide/caustic potash.*

Stage (a) is *always* an essential preliminary, since the normal vapour-pressure/temperature relations do not hold in this type of analysis, and dry gases only should be admitted to capillary burettes. When it is necessary to measure the concentration of water vapour in a gas sample, then some other method of analysis must be employed (see, for instance, the absorption methods used in Combustion Analysis, p. 65).

METHOD. The necessary reagents are prepared on the platinum wires, as described on pp. 513–515, loaded into the guides in the rotating clamp, and immersed under the surface of the mercury so that undue exposure to the atmosphere is prevented.

Reagents	Water Vapour	Carbon Dioxide	Oxygen	Olefines
Phosphorus pentoxide	Quantitative absorption*			
Sulphuric acid in porous bead	Quantitative absorption*			Partial absorption
Potassium hydroxide bead	Quantitative absorption*	Quantitative absorption*		
Yellow phosphorus bead			Quantitative absorption*	Interferes
Chromous sulphate solution in porous bead			Quantitative absorption*	
Fuming sulphuric acid in porous bead	Quantitative absorption			Quantitative absorption*
Cupric oxide+1% ferric oxide				Oxidised completely
Cuprous chloride+potassium hydroxide bead		Interferes	May interfere	Specific for acetylene in presence of methane*
Silver oxide bead		Interferes		
Copper oxide+potassium hydroxide bead		Interferes		
Potassium bisulphate bead				
Cadmium acetate solution in porous bead				
Hot wire combustion in excess hydrogen			Quantitative reduction*	
Hot wire combustion in excess oxygen				Oxidised completely

* Indicates the intended use

CAPILLARY BURETTE METHODS OF GAS ANALYSIS

Saturated Hydrocarbons	Carbon Monoxide	Hydrogen	Sulphur Dioxide and Acid Gases	Ammonia	Hydrogen Sulphide
				Some absorption	
				Quantitative absorption*	
			Some absorption		
Interferes		May interfere			
			Interferes		
Some members absorbed				Quantitative absorption	
Methane* quantitatively oxidised (600°)	Interferes	Oxidised completely	Interferes		Interferes
	May interfere		Interferes		Interferes
	Quantitative absorption*	Interferes slightly		Interferes	Interferes
		Oxidised completely*	Interferes		Interferes
				Quantitative absorption*	
					Quantitative absorption*
			Sulphur dioxide reduced		
Oxidised completely	Oxidised completely	Oxidised completely*		Interferes	Interferes

of the particular reagent.

A number of gas-holders are filled with mercury, inverted in the trough, and clipped into position. Any small trapped air bubbles may be removed with a bent capillary pipette.

The burette is then inspected to make sure that it is completely full of mercury, and the table carrying the trough is screwed up until the gas-holders can be rotated over the tip of the burette.

The sample is introduced into the gas-holder, and the loop carrying the phosphorus pentoxide is carefully manipulated to enter the gas space, without touching the walls of the tube. After about 5 minutes the bead is removed similarly, the gas-holder is then rotated over the burette, and the table is lowered until the tip of the burette projects into the bubble and finally touches the top of the gas-holder. By unscrewing the plunger of the burette reservoir C all the gas is drawn into the burette, and is followed by a column of mercury. This column of mercury must be so adjusted that the meniscus above the gas in the burette is in horizontal alignment with the mercury in the main trough T. To effect this, the mercury level is brought to the zero mark on the burette and the trough is adjusted to bring its mercury surface up to the tip of the fixed ivory pointer P (fig. VI.1).

During this adjustment it is essential to tap the burette continuously to prevent the mercury from sticking to the dry glass. Four or five adjustments should be made, and the length of the gas bubble measured each time, the mean being taken as the true length.

When the first measurement has been made (giving the volume of the dry gas at atmospheric pressure) the temperature and the barometric pressure should also be noted.

The second gas-holder is now rotated into position and the gas is expelled into it from the burette. This gas-holder is then removed so that the caustic potash pellet can be inserted. Since at this stage the gas is dry, the utmost care must be taken to see that the glass rod of the reagent-holder does not touch the gas-holder or enter the gas space. If this precaution is not observed then there is considerable risk of carrying adsorbed moisture and air into the sample, thus vitiating the whole analysis.

This routine is repeated for each of the other reagents in turn until only hydrogen and nitrogen are left.*

For the removal of hydrogen the bubble of gas is expelled into a clean gas-holder, the table is lowered, and the holder is moved from its position above the burette jet. A small iron block in which has been drilled a blind hole of such a diameter that it takes the gas-holder easily is lowered under the surface of the mercury in the trough, and the gas-holder is then inserted in the hole. The block and gas-holder are removed together and placed

* NOTE.—If samples rich in hydrogen are being analysed then some absorption of hydrogen may occur during the removal of oxygen by the phosphorus bead (p. 514). In such a case it is advisable to remove hydrogen *before* absorbing oxygen.

in the mercury trough of the heater stand shown in fig.VI.3. When the gas-holder has been clipped into position inside the heater a copper oxide/ caustic potash bead (p. 515) is inserted into the gas in the usual manner, the heater is switched on until the lead is *partly* melted, and the assembly is then maintained at this steady temperature for 15 minutes. Finally the heater is removed, and when the gas-holder has cooled down it is retransferred to the main mercury trough for measurement of the volume of the residual gas.

SOURCES OF ERROR.

1. The surface of the mercury in the trough soon becomes covered with dust, which is liable to find its way into the burette where it can cause endless trouble. Blacet and Leighton recommend the use of a capillary pipette attached to a filter pump as a miniature "vacuum cleaner." A mercury trap should be fitted in the circuit, since a small amount of mercury is inevitably carried over.

2. All measurements must be made with dry gas. Hence if moist reagents or reactions (e.g. combustions) which produce water are used, then the sample must be treated with a bead of phosphorus pentoxide before the gas volume is measured.

3. Spark reactions should be avoided if possible, since, if nitrogen is present in the gas mixture, oxides of nitrogen, which can cause trouble, may be formed.

4. Ammonia is very strongly adsorbed on to glass. When analysing for this constituent the gas should be drawn back into the burette after the first absorption, left for a minute so that desorption from the burette walls can take place, and then passed back into the gas-holder for retreatment with the absorbing agent. If the initial concentration of ammonia is high then a second desorption and reabsorption should be carried out.

(b) T. C. Sutton's Technique*

The apparatus described in 1938 by T. C. Sutton is also intended for use with dry samples and solid reagents. From fig. VI.5 (p. 520) it will be seen that Sutton's apparatus differs from that of Blacet and Leighton in several important respects: (i) the capillary burette is arranged horizontally; (ii) the absorption vessel is integral with the burette; (iii) a thermobarometer is used to compensate for changes in temperature or barometric pressure occurring during an analysis. These features are designed to simplify the procedure so that relatively unskilled personnel can obtain good results after a minimum of training.

Though Sutton does not claim such great precision as do Blacet and his

* Sutton, T. C., *J. Sci. Inst.*, 1938, **15**, 133.

colleagues, there is no fundamental reason why the technique should not give equal or better accuracy than other similar methods. For samples of the order of 0·2 ml. the readings are reproducible within 0·25%.

PRINCIPLES. The sample for analysis is introduced into the enlarged end of a capillary burette and from there can be drawn into the main calibrated portion for measurement. A similar volume of air is introduced into the thermo-barometer and the length of this bubble also noted.

The various constituents of the gas mixture are absorbed in turn by expelling the gas into the enlarged end of the burette and introducing a suitable solid reagent on a platinum loop or porous bead.

Changes in temperature, barometric pressure, or the relative positions of the various parts of the apparatus are compensated for by adding or
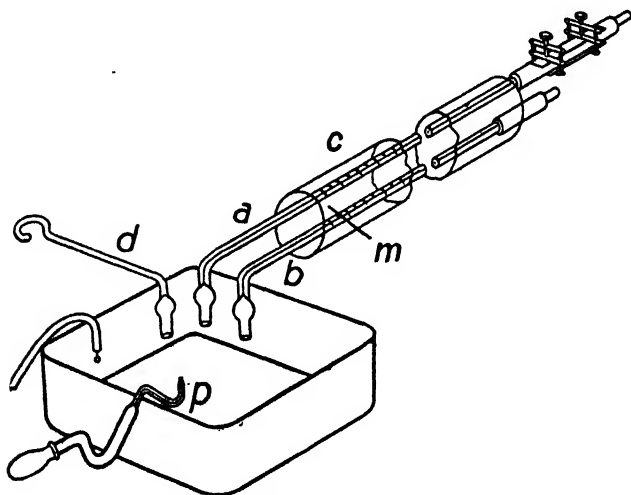
Fig. VI.5.   T. C. Sutton's Apparatus for Micro Gas Analysis.

removing mercury to or from the trough until the bubble in the thermo-barometer regains its initial length.

APPARATUS. The measuring burette a (fig. VI.5) is made from uniform capillary tubing of 0·5 mm. bore, and is about 60 cm. long with the last 3 cm. bent at right-angles. A piece of 4 mm. bore tubing, also 3 cm. long, is fused to this vertical limb and forms the absorption vessel.

The remote end of the burette is fitted with a device for advancing the mercury column. An exactly similar tube b is arranged parallel to the measuring burette to serve as the thermo-barometer, but since there is no need for precise control of the mercury in this case, its further end is closed only by a stout rubber teat. Both burette and thermo-barometer are calibrated over their horizontal portions and are enclosed in a common

air-jacket c. Alternatively, the two capillaries may carry reference marks at m only, while the divisions can be etched on the outer jacket. This second method makes the reading easier, but, unless the air-jacket is very narrow, is apt to introduce parallax error.

The burette assembly is rigidly clamped in a horizontal position with the vertical portion dipping into a trough of mercury, the level of which is so adjusted that the junction of the absorption vessel and the capillary is just visible above the surface.

Suitable auxiliary gas-holders, consisting of 4 mm. tubing fused to the end of glass rods, are shown at d, while p is a gas pipette constructed from capillary tubing with an enlargement blown in the centre and fitted with a stout rubber teat (compare fig. VI.6, D). It serves both for the transfer of gas to and from the burette and for adjusting the level of mercury in the trough. Apart from the shape of the holders (figs. VI.6, A, VI.6, B) the
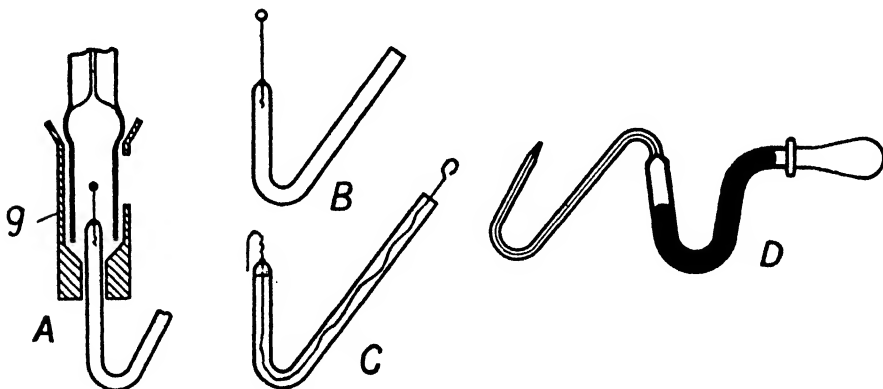
Fig. VI.6. T. C. Sutton's Apparatus.

A, reagent-holder inserted in absorption chamber with the aid of the steel guide. B, reagent-holder. C, conductor for "hot wire" reactions. D, gas pipette.

reagents are prepared and used in the same manner as is described on pp. 513–515.

The steel guide g, which enables the reagent-holders to be inserted in the absorption vessel of the burette without contaminating the sides, is an essential feature of Sutton's apparatus.

ANALYTICAL PROCEDURE. The requisite reagents are prepared and immersed in the mercury in the trough. The gas sample is then transferred to a gas-holder previously filled with mercury. The sample is then dried with a phosphorus pentoxide bead as follows: one of the steel guides g is slipped over the platinum loop containing the reagent until it fits neatly on the glass rod of the holder (see fig. VI.6, A). The holder, with the steel guide, is kept under the surface of the mercury to avoid trapping air bubbles, and is moved until it can be placed over the gas container. The flared end of

the guide makes this a simple matter, and the reagent should enter the gas space cleanly.

When the sample is dry, a gas pipette* (fig. VI.6, D) is filled with mercury, making sure that *all* air bubbles have been expelled, and the jet is inserted into the gas space. Very gentle squeezing of the teat then expels a little mercury, and on releasing the pressure the gas is drawn into the pipette followed by a column of mercury. The dry gas is then transferred from the pipette to the reaction chamber on the measuring burette and from there drawn into the burette by unscrewing the knob until it is just beyond the fixed mark m (fig. VI.5). A similar amount of the same *dry* air is drawn into the thermo-barometer and then the mercury is advanced in the burette until the meniscus just approaches the fixed mark m. This routine of drawing the gas beyond the reference mark and then moving it back exactly to the mark must be followed before each measurement to ensure that the pressure of the gas is just sufficient to overcome the resistance of the mercury meniscus in fig. VI.6, D. Only by adopting this procedure can highly reproducible measurements be made.

At this stage the length of the gas column in each of the tubes should be noted carefully; the gas should then be expelled from the burette and the measurement repeated as a check. Finally, the gas is again expelled into the absorption chamber and the first reagent is inserted with the help of the steel guide. After absorption of the first constituent, the gas is returned to the burette and re-measured. If the length of the air bubble in the thermo-barometer has changed, it must be readjusted to its original length by adding or removing mercury to or from the trough, *before* the gas in the burette is measured. The gas pipette p is convenient for this purpose. The same measurement procedure is repeated after the removal of each component gas.

* The gas pipettes used for transfer of *dried* gas should be kept over phosphorus pentoxide in a desiccator until required.

B. THE CONSTANT-VOLUME METHOD OF R. SPENCE*

This method, which is of recent introduction, compares favourably with the constant-pressure techniques both on the score of convenience and accuracy. The apparatus, though rather more elaborate than the capillary burettes already described, is not difficult to construct by anyone with average glass-working skill and can be used for any of the normal types of gas analysis.

Its main advantage over the constant-pressure methods is its direct applicability to cases in which the gas to be analysed is contained in a system at low pressure, but, in addition, the method suffers less from adsorption errors.

The order of accuracy obtainable is 0·1% for samples of about 50 cu. mm.

PRINCIPLES. The sample for analysis is introduced into the apparatus directly from a system at low pressure or from a gas-holder at atmospheric pressure. It is then adjusted to *constant volume* and its pressure is measured by a mercury manometer. By manipulating a mercury reservoir, the gas is caused to circulate round a capillary loop containing a solid reagent, the flow being maintained unidirectional by means of non-return valves. After the first constituent has been absorbed the sample is returned to the gas burette, readjusted to its original volume, and the new pressure is measured. A fresh loop containing a reagent suitable for the absorption of the next constituent is then substituted and the process is repeated.

From the differences in pressure so obtained, the percentage composition of the sample can be calculated.

APPARATUS. The essential feature of the apparatus, which is illustrated in figs. VI.7–9 (pp. 524–525), is the capillary absorption loop (fig. VI.7, *L*) shown in detail in fig. VI.8.

This is constructed as follows:

Pyrex quill tubing is first drawn to a suitable taper from which the sockets of the small ground joints can be made. These tapers are then cut about 7 mm. long and fused to the ends of short lengths of capillary tubing, which should have a bore between 0·5 and 0·25 mm. The cone portion of each joint is prepared by grinding the ends of further lengths of capillary to approximately the correct taper on an emery wheel, and the joints are completed by grinding the cone and socket portions together carefully with carborundum paste.† Finally, the tubes are formed to the shape shown in the diagram. The top joint serves as the reaction chamber, so that a space of 3–5 mm. should be left during the construction. Simple non-return valves (fig. VI.9) are accommodated between the ends of the capillaries in the other joints, and in these parts the space required is only about 0·2 mm.

* Spence, R., *J. Chem. Soc.*, 1940, 1300.
† A very accurate fit is not essential since the joints must be sealed with picein wax before use.
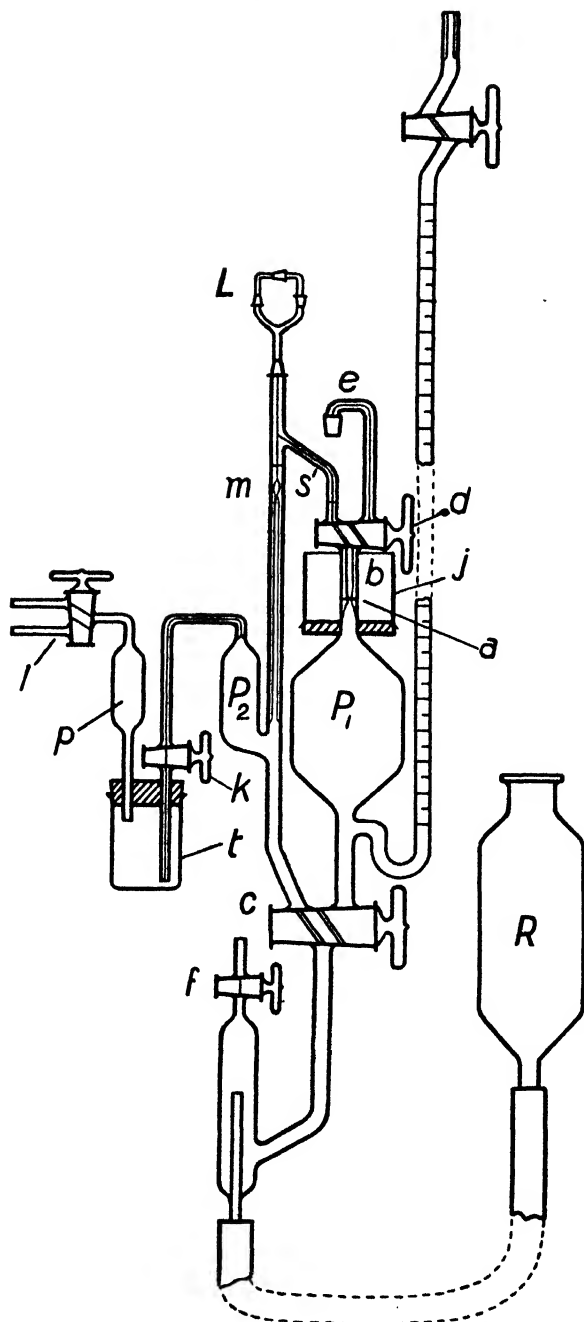
Fig. VI.7.   R. Spence's Apparatus for Constant Volume Micro Gas Analysis.   General arrangement of complete assembly.

The valves proper are made by cutting thin laminae of mica to a suitable size and inserting them in the joint before assembly.

A number of these loops are needed—one for each reagent. In addition, a loop of the type illustrated in fig.VI.8, *b*, constructed from a 1·5 mm. bore capillary, containing a copper spiral and wound on the outside with a heating coil, is required for oxygen absorption (see below, p. 526). Similar loops fitted with electrodes or resistance elements can be prepared for "spark" or "hot wire" reactions.

When not in use, each loop is closed by a suitable stopper.
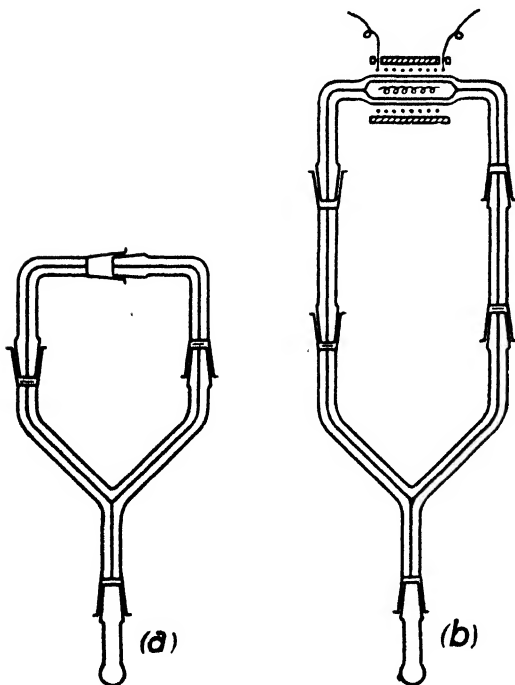
Fig. VI.8. R. Spence's Apparatus.
Capillary absorption loops.

Fig. VI.9. R. Spence's Apparatus.
Valve details.

The remainder of the apparatus is shown in fig. VI.7. It consists of two Töpler pumps P_1 and P_2 arranged in parallel, and capable of being connected to the common reservoir R by the two-way cock c. A manometer and scale are attached to the larger Töpler pump, whilst the absorption loop is fitted to the smaller one, as shown. The wide-bore capillary is constricted at m to stop mercury from surging into the loop; the constriction also carries a reference line.

The gas burette *b* is a length of capillary tubing of 1·5 mm. bore placed immediately below the two-way cock *d*, and is surrounded by a water-jacket *j*.

About 4 cm. below the cock the tube is constricted to 0·5 mm. and has a reference line a etched on the outside. The capacity of the burette, which should be about 70 cu. mm., must be known accurately.

The volume of the large bulb P_1 must also be known. Both volumes are best obtained during construction in the manner described for calibration of the Van Slyke apparatus (p. 538).

REAGENTS. In general, the solid reagents described on pp. 513–515 may be used with success, though in a few cases alternative compositions are preferable.

It is essential to remove water as it is formed, and hence it is advantageous to incorporate dehydrating agents into some of the absorption loops.

Hydrogen and *oxygen* can be oxidised together at the surface of hot copper oxide in a loop of the type shown in fig. VI.8, *b*, with beads of phosphorus pentoxide contained in the upper joints. The hydrogen can then be measured directly by removal as water, and a new loop containing caustic potash can then be fitted to the system for the absorption of the carbon dioxide produced from the carbon monoxide.

For the oxidation of *paraffins* such as *methane* and *ethane* Spence recommends the use of Arneil's catalyst,* which is prepared as follows: 99 parts by weight of cupric oxide are mixed intimately with 1 part of ferric oxide and 20% of finely ground kaolin is added. The mixture is made into a stiff paste with water and is allowed to dry. When quite dry, the hard mass is broken into small pellets and loaded into a loop of type *b* (fig. VI.8), the upper part of which is made of quartz and is wound with a resistance element capable of raising the temperature of the mass to 600° C. The catalyst is heated and evacuated thoroughly, after which the gases can be circulated through it. With fresh catalyst, combustion is complete in a few minutes; the catalyst may be re-activated by circulating oxygen over the hot mass.

By this method it is a simple matter *to determine the relative amounts of methane and ethane in a mixture of the two,* since one volume of methane produces one volume of carbon dioxide, whereas one volume of ethane produces two volumes of carbon dioxide. Therefore, if the water vapour is absorbed as formed by phosphorus pentoxide (see above) any *increase* of pressure is directly equivalent to the ethane present. The methane is determined from the total quantity of carbon dioxide formed by subtracting twice the volume estimated as ethane.

ANALYTICAL PROCEDURE. Before commencing an analysis, the large Töpler P_1 (fig. VI.7) is freed from adsorbed gas by evacuating it several times and expelling any desorbed gas through the smaller Töpler P_2. The mercury level is then allowed to rise to the reference mark on the gas burette b and the zero pressure reading, P_0, is taken. The mercury reservoir is

* Arneil, A., *J. Soc. Chem. Ind.*, 1934, **53**, 89.

then raised until the burette, the upper two-way cock *d*, and the side-arm *s'* are completely full of mercury. The cock can then be closed and the reservoir parked.

Collecting the Sample. If the gas to be analysed is in a system at low pressure, this latter is attached to the standard joint *e*, and a sample is drawn into the burette by evacuating the large Töpler pump. If however the sample is at atmospheric pressure, it should be contained in a simple gas-holder of the type already mentioned (*d*, p. 520, or *g*, Fig. VI.2, p. 511). The trough and gas-holder are then brought close to the standard joint *e*, which is immersed under the surface of the mercury. By means of a pipette of the type described on p. 521, the sample can be transferred from the gas-holder to the inside of the standard joint, and from there drawn into the burette. It is automatically followed by a column of mercury which seals the capillary and the bore of the tap.

Measurement and Absorption of Constituents. After the sample has been transferred to the burette, it is expanded to a pressure of less than 1 mm. by lowering the mercury in the large Töpler pump. This ensures desorption of gas from the walls of the apparatus. The mercury is then allowed to rise again until the meniscus is in line with the reference mark *a* and the pressure in P_1 is noted.

A capillary loop, containing the first reagent, is now attached to the smaller Töpler pump, and the joint is sealed with picein wax to ensure a vacuum-tight system. Next the mercury in the smaller Töpler is lowered well below the bulb cock *c* and a rough vacuum (e.g. that from a filter-pump) is applied at *b* so that the pump and loop are both partly evacuated. Finally the loop is evacuated completely by a few strokes of the small Töpler pump. When operating this pump it is necessary to close the cock *k* until the extracted gas is trapped in the capillary immediately above it. The cock can then be opened cautiously and the gas bubble, together with a little mercury, allowed to pass through into trap *t*.

When the loop is completely vacuous, mercury is allowed to rise in the small Töpler pump until the reference mark *m* below the loop joint is reached. Cock *C* is then turned to put the bulb of the larger Töpler in communication with the reservoir, which is again lowered to expand the sample. After about 30 seconds the gas is re-compressed into the burette and the pressure P_1 is checked. The top cock *d* is opened next and the gas is allowed to pass into the absorption loop, through which it is circulated by alternately raising and lowering the mercury reservoir. The movement should be controlled so that the mercury oscillates in the vertical part of the capillary above the cock with an amplitude of about 2 cm. If a subsidiary reference mark is placed on this capillary, the absorption can be followed without having to return the sample to the burette.

When absorption is complete, the mercury is lowered to the mark below

the large bulb and the gas is left in the expanded state for about 1 minute to ensure desorption. Finally, cock d is closed, the sample is re-compressed to the mark on the micro-burette, and the new pressure P_2 is observed.

It will be appreciated that P_2, as observed, will be a little too low (about 0·1%), due to the small amount of gas which is left in the loop when the sample is expanded prior to reading the pressure. A correction is therefore made by multiplying $(P_2 - P_0)$ by the ratio

$$\frac{\text{Total volume of loop, burette, and Töpler bulb}}{\text{Volume of burette and Töpler bulb}}$$

to give P_2 corr.

Since the volume of each loop will be different according to its precise dimensions and the amount of reagent which it contains, it is convenient to determine the volume immediately an absorption has been carried out.

The method is as follows: After noting P_2 the residual gas in the loop is removed by three or four strokes of the small Töpler, and the mercury is readjusted to the mark below the loop. The gas in the burette is next expanded to desorb, and cock d is opened to the loop. Then mercury is allowed to rise in the large Töpler pump until the line on the burette is reached, when a pressure reading P_3 is made. The ratio $(P_2 - P_0)/(P_3 - P_0)$ is proportional to:

$$\frac{\text{Volume of burette} + \text{loop}}{\text{Volume of burette alone}}.$$

Since the volume of the burette is known, that of the loop can easily be determined.

The percentage of the absorbable constituent in the sample is then given by the equation:

$$100 \times \frac{(P_1 - P_0) - (P_2 \text{ corr.})}{(P_1 - P_0)}.$$

When the first constituent has been removed from the gas mixture, it is necessary to substitute a fresh loop containing another reagent. The change is effected as follows: The lower cock c is turned and mercury in the small Töpler pump is lowered well below the bulb. After closing the cock, *dry* air is cautiously allowed in to the system, via cock l, and the phosphorus pentoxide tube p, at the same time causing most of the mercury in trap t to be returned to the Töpler. The first loop is then removed and its open end is at once sealed with a stopper. Another loop is substituted in its place and the whole routine is repeated as described above.

With easily absorbable gases, each cycle can be completed in about 5 minutes.

PRECAUTIONS.

1. If a change of temperature occurs during the interval between the taking of the initial reading P_1 and the completion of the analysis, then all

the subsequent pressure measurements must be corrected in accordance with the ordinary gas law.

2. The internal glass surfaces must be kept perfectly dry. They may be dried initially by drawing into the system a small quantity of air from an adequate drying train and then leaving it in an expanded state for a short time. After expelling this air the process should be repeated once or twice. The manometer may be dried with trimethylene glycol (see pp. 539–540).

3. Greasing of the cocks requires care (see p. 539). An excess of lubricant must be avoided, to ensure that none finds its way into the capillaries.

4. Mercury is very apt to stick to dry glass, so that care must be exercised to avoid breaking the column when passing mercury from a narrow capillary to a wider portion of the tube. On no account should mercury be allowed to enter the absorption loops.

C. THE GLYCERINE ENTRAINED BUBBLE METHOD

(Application to Analysis of Gas Bubbles in Glass)

The analysis of the various gases occurring as minute bubbles in optical glass is a matter of technical importance, and in recent years the bubble method of Krogh* has been adapted to this purpose, first by Enss† and later, quite independently, by Woano‡ and by Price and Woods.§ A similar technique could probably be applied to problems in metallurgy, provided that the gas bubbles can be located by X-rays or other means.

Since general techniques of gas measurement and of absorption of individual components are involved, this simple micro-chemical procedure deserves wider recognition.

PRINCIPLES. The gas bubble is removed from the glass while the latter is submerged in glycerine.   The rising bubble is caught on the underside of a microscope slide and its diameter is measured with the eyepiece scale of a microscope.   By means of suitable pipettes, the bubble is then transferred successively to various reagents dissolved in glycerine or water, and is remeasured after the absorption of each constituent.

It is assumed that the bubble is truly spherical.   Hence if the original volume $V = \dfrac{\pi D^3}{6}$, and the volume after an absorption $V' = \dfrac{\pi D'^3}{6}$, then the percentage of gas absorbed

$$= 100 \left(\frac{V - V'}{V} \right) = 100 \left[1 - \left(\frac{D'}{D} \right)^3 \right].$$

Bubbles with diameters of from 0·2 mm. to 3·0 mm. can be dealt with quite readily.

APPARATUS.

1. A good low-power microscope fitted with a mechanical stage and eyepiece micrometer.

2. Pipettes, illustrated in fig. VI.10.

3. Small copper troughs 3 in. × ¾ in. × ½ in., fitted with two narrow shelves on which a ¾ in. length of ordinary 3 in. × 1 in. microscope slide can be supported.   A thick ¾ in. circular coverslip is cemented with Chatterton's compound into a ½ in. diameter hole in the bottom of each box.   The boxes are painted black to facilitate observation, and when not in use are covered by a tightly fitting lid to exclude moisture from the glycerine.

4. A good magnifying glass, dissecting needles, and pointed forceps.

* Krogh, A., Skand. Archiv. Physiol., 1908, 20, 279.
† Enss, J., Sprechsaal f. Keramik. Glas, Email., 1933, 66, 662.
‡ Woano, W. G., Optiko. Mekanik. Promyschlenost., 1937, 7, 14.
§ Price, W. B., and Woods, L., Analyst, 1944, 69, 117.

For glass analysis some means of grinding the glass blocks and a pressure clamp for piercing the surface of the glass must also be available. Suitable apparatus has been described by Price and Woods.*

REAGENTS.

For carbon dioxide. Dissolve 10 g. of solid caustic potash in 100 ml. of anhydrous glycerine and store in a sealed vessel.

For hydrogen sulphide. Dissolve 10 g. of cadmium acetate in 100 ml. of anhydrous glycerine and store as above.

For nitric oxide.

(i) Saturated potassium permanganate solution, acidified with sulphuric acid.

(ii) Saturated ferrous sulphate solution, acidified with dilute sulphuric acid.
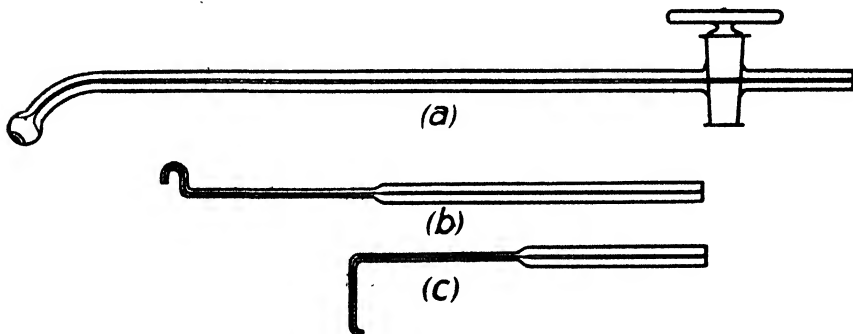
Fig. VI.10. Pipettes—Glycerine Bubble Apparatus (after Price and Woods).*

For oxygen.

(i) Dissolve 0·5 g. of fresh sodium hydrosulphite ($Na_2S_2O_4$) in 2·0 ml. of 13·5% aqueous potassium hydroxide.

(ii) Alkaline pyrogallol.

(iii) Chromous sulphate solution (p. 514).

For carbon monoxide. Dissolve 11·25 g. of ammonium chloride in a mixture of 14 ml. concentrated ammonia and 33 ml. of water, and add 9 g. of freshly prepared cuprous chloride. Store in a sealed bottle containing a spiral of clean copper wire (see p. 548).

For hydrogen. Colloidal palladium suspended in saturated sodium picrate solution. Prepare the colloidal palladium as follows: Boil 0·5 g. of palladium chloride in a mixture of 8 ml. of water and 2·8 ml. of 2N hydrochloric acid until all has dissolved. After cooling add this solution slowly to a solution

* Price, W. B., and Woods, L., *Analyst*, 1944, **69**, 117.

of 0·65 g. of sodium protalbinate* in 16 ml. of water and 10 ml. of $2N$ sodium hydroxide. Reduce the product by slowly adding 5 ml. of 50% aqueous hydrazine hydrate. Dialyse the resulting black solution for 3 days and finally evaporate to dryness in a vacuum desiccator to room temperature. 0·06 g. of colloidal palladium should be dissolved in 4·35 ml. of sodium picrate solution.

For phosphine. Dissolve 15·6 g. of anhydrous copper sulphate in 100 ml. glycerine and add 1 ml. of concentrated sulphuric acid.

ANALYTICAL PROCEDURE.

1. *Collection and Measurement of Sample.* The glass specimen containing the bubbles is cut to fit easily in the trough, and the surface nearest to the selected bubble is then ground until a wall of only about 1/1,000 in. remains.

The prepared block is submerged under the glycerine in a trough, so that the bubble is uppermost, taking care to avoid trapping any air. A shortened microscope slide is next placed in position on the shelf and the thin glass membrane is pierced with a bent dissecting needle, so that the gas can rise to the underside of the slide. The diameter of the bubble is then measured in terms of scale divisions, after which the bubble is sucked into a capillary pipette of about 0·5 mm. bore and transferred to the first absorbent.

2. *Absorption of Component Gases.* The reagents, in glycerine, are contained in a separate small vessel in which an open glass tube, about 1 cm. diameter and 1·5 cm. long, is submerged. The bubble is transferred to the inside of this tube, which is then rolled so that the gas bubble is moved to the lower inside surface of the cylinder and afterwards can rise through the reagent until it reaches the upper inside surface. This procedure, which facilitates reaction, is repeated as often as necessary, but one or two excursions are usually sufficient.

Absorption by means of aqueous reagents is effected in a pipette of the type shown in fig. VI.10, *a*. This is made from capillary tubing of about 1·5 cm. bore, and is fitted with a tap 5 cm. from one end. The longer arm, bent as shown, measures about 25 cm. and terminates in a 5 mm. bulb in one side of which is blown a circular opening about 3 mm. diameter.

The pipette is filled with the appropriate reagent by attaching a rubber teat to the end nearest the tap and aspirating the fluid slowly through the open bulb, exercising the utmost care in order to avoid any trapping of minute air bubbles, particularly when using dark solutions such as colloidal palladium (p. 531). The quantity of reagent contained in the pipette should be such that a meniscus is formed across the 3 mm. opening. When full, the tube is clamped horizontally with the opening in the bulb downwards, and the gas bubble is introduced by a pipette of type *c* (fig. VI.10). By

* Paal, C., *Ber.*, 1902, **35**, 2195.

tilting the tube the bubble is caused to rise towards the tap, and then is returned by further tilting in the opposite direction until it re-enters the bulb, from which it can be removed by a pipette of the shape shown in fig. VI.10, *b*.

In most cases one passage through the reagent is sufficient. The bubble should then be rinsed in clean glycerine (using the submerged cylinder technique) so that traces of absorbent are not carried into the trough.

Special Procedure for Analysis of Soluble Gases. Whilst many gases such as nitrogen, hydrogen, hydrocarbons, carbon monoxide, and phosphine are not appreciably soluble in glycerine and yield bubbles which do not alter appreciably in size during several minutes, others, such as sulphur dioxide and carbon dioxide, easily dissolve and so cannot be estimated by the general procedure given above.

Bayer* has suggested that the rate of solution of a gas can often be used for its estimation.

Thus for a bubble initially of 3·5 mm. diameter:

Sulphur dioxide dissolves completely in 40 seconds; *hydrogen sulphide* loses 50% of its volume in 2 minutes; *carbon dioxide* loses 50% of its volume in 10 minutes; whilst *water vapour* dissolves at once. So too does *nitric oxide*, which is promptly converted to nitrogen peroxide by the oxygen dissolved in the glycerol.

Hence, if qualitative tests for the presence of these soluble gases have previously been applied, observation of the bubble size under the microscope during the first 10 minutes after collection will yield sufficient information for the approximate computation of the chemical composition.

Suitable tests for these soluble gases are given in Table IV of Part IV (pp. 378–379). The colorimetric analyses described in that section can of course be carried out on aliquot parts of the final solution in glycerine or water.

* Bayer, F., "Gasanalyse" (F. Enke, Stuttgart, 1941).

MANOMETRIC TECHNIQUES OF MICRO-ANALYSIS

A. USES OF THE MANOMETRIC VAN SLYKE APPARATUS

Introduction

There are two types of Van Slyke apparatus in current use: the original volumetric pattern (fig. VI.11) and the closed manometric pattern (fig. VI.12). The prototype, devised by Van Slyke in 1917, is still widely used in clinical laboratories, but has been largely superseded for research purposes by the manometric pattern on account of the latter's greater precision and versatility, though it does not seem to be widely known outside the biochemical field.

It frequently happens that analyses can be made with the Van Slyke apparatus far more accurately than by conventional methods. Such a case is the determination of carbon in non-volatile organic compounds (pp. 551–556).

The manipulation of the Van Slyke apparatus requires some practice before the highest precision can be achieved, though if careful attention is paid to the details of the technique, very reproducible and highly accurate results can be obtained.
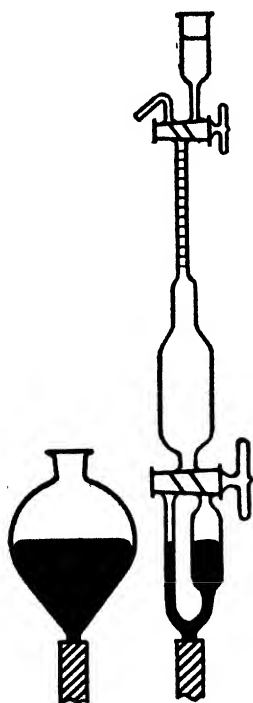
Fig. VI.11. Van Slyke Volumetric Apparatus.

Principle of Operation (Both Types)

The solution to be analysed is introduced into an extraction chamber, preceded or followed by the necessary reagents. The mixture is then subjected to a vacuum by means of a mercury column and reservoir, and the extraction of dissolved gases is completed by shaking for a suitable time. At the end of the time the gases are either brought to atmospheric pressure and their volume measured (volumetric apparatus) or brought to constant volume and their pressure measured (manometric apparatus). Absorbable gases are removed singly by introducing suitable reagents into the chamber, and further measurements are made as needed. The relative concentration of the various gases present can be obtained by calculation or by reference to prepared tables.

534
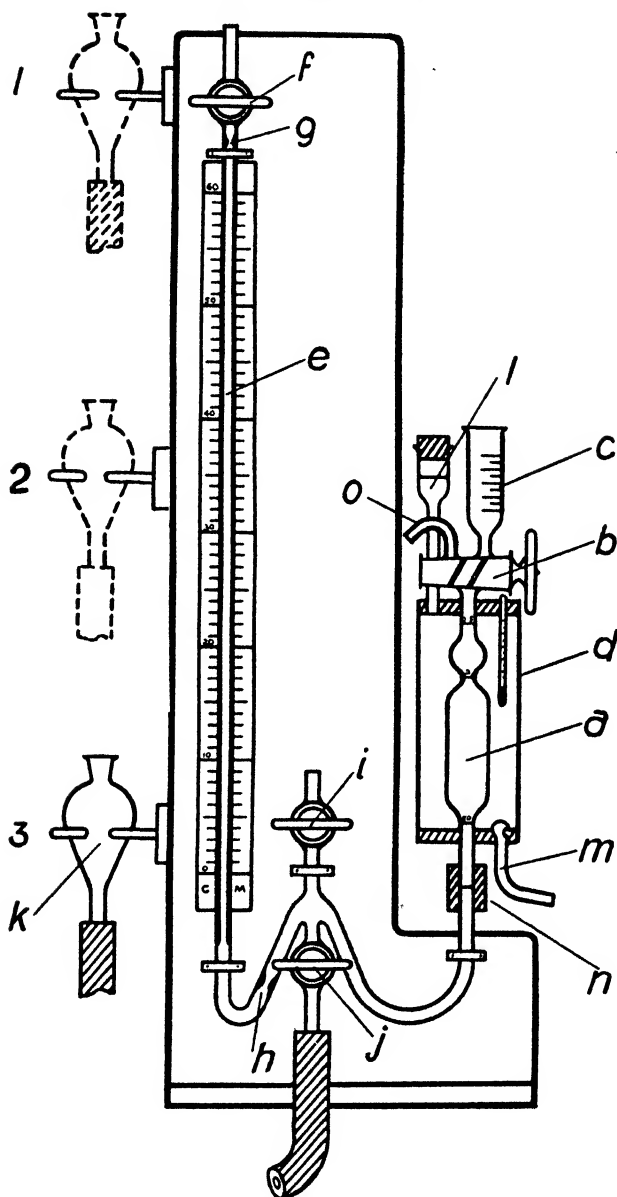
Fig. VI.12. Van Slyke Manometric Apparatus.

The Manometric Apparatus

General Design. Fig. VI.12 is a diagram of the usual form of manometric apparatus. The extraction chamber a is of 50 ml. capacity from the key of the upper two-way cock b to the lowest graduation, further graduations

being provided at 2 ml. and 0·5 ml. volumes. The cup c has a capacity of 10 ml., graduated at 0·5 ml. intervals, and is fused to the cock by a short capillary tube. A water-jacket d is fitted round the chamber by two rubber discs, the upper one being divided in halves, one of which carries a short thermometer reading to 30° C. The manometer e, about 65 cm. long, is closed at the top by a cock f, below which is a constriction g to prevent the mercury impacting too heavily against the tap. The tube is graduated at 1 mm. intervals and numbered at each centimetre division. A second choke h is formed in the left-hand U-tube to prevent violent oscillation of the mercury. The cock i serves to release any air collected in the trap-tube above the cross-joint and the large cock j is used to control the flow of mercury to and from the reservoir k. The reservoir is connected to cock j by a length of pressure tubing.

The whole assembly is fitted to a wooden stand and provided with a shaking mechanism attached to the chamber water-jacket.

Constructional Details

A. Glass Cocks. These should be the "precision-ground" type sold for high-vacuum work, since successful analysis depends on their gas-tightness. The two bores (1·2–1·4 mm. diameter) in the key of cock b (fig. VI.12) should meet the capillary tubing truly and should not be countersunk. The large control cock j should be of 3–4 mm. bore, while the remaining cocks i and f can be about 1½ mm. in bore.

B. The Extraction Chamber. Two types are shown in figs. VI.13 and VI.14. Fig. VI.14 is the normal pattern, while fig. VI.13 is necessary for such analyses as Example 3 (pp. 551–556). There is, of course, no reason why other volumes should not be used if required for special purposes. The optimum diameter of the glass tubing at the points of calibration is as follows:

Graduation	Diameter of Tube (bore)
0·5 ml.	4 mm.
2·0 ml.	4 mm.
10·0 ml.	6 mm.
50·0 ml.	6 mm.

Although not very critical, the dimensions used should not vary greatly from those given, e.g. if the tube from the cock b (figs. VI.12 and VI.13) to the 0·5 ml. graduation is much less than 4 mm. difficulty may occur in removing a column of liquid trapped in it during analysis, while if greater, the graduation line is so close to the rubber bung closing the water-jacket that observation of the meniscus is extremely difficult.

The cup c above the upper cock should be made with a well-rounded bottom and the capillary below should not be more than 1 cm. long.

The side-arm o must not be bent towards the barrel of the cock nearer

than 1 cm., otherwise it will not be possible to fit it with heavy tubing when required.

C. The Water-jacket. This must be wide enough to pass over the extraction chamber (when the key of the upper stop-cock is removed) to allow easy access for cleaning, etc. Many commercial instruments fail badly in this respect. The ends of the jacket should be finished square, since if they are flared the rubber discs may slip out.

In the illustration (fig. VI.12) the jacket is shown fitted with a closed thistle funnel *l* and drain tube *m*. Commercial instruments are not so fitted, with
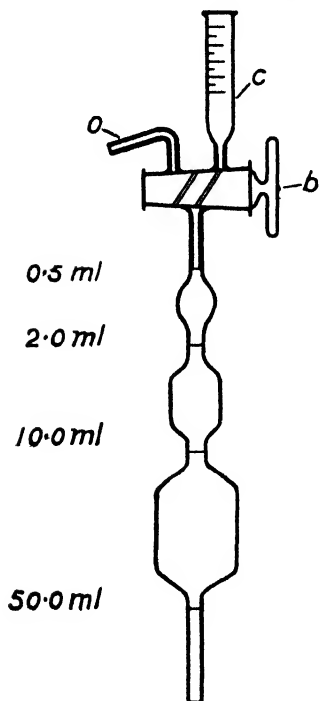
Fig. VI.13. Van Slyke Apparatus
Extraction Chamber. Special
Pattern.
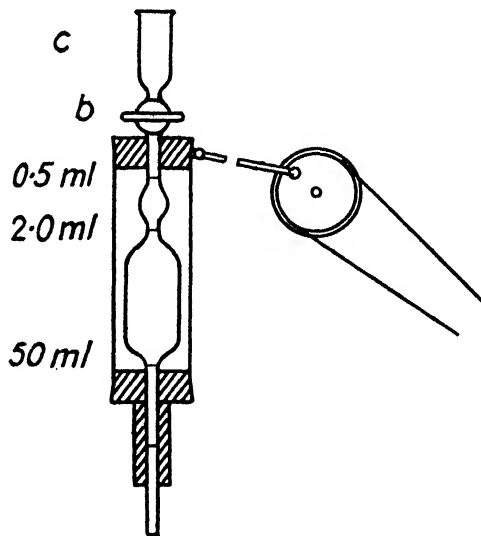
Fig. VI.14. Van Slyke Apparatus. Extraction
Chamber. Normal Pattern.

the result that the air space is immediately under the top rubber and after shaking a layer of froth prevents observation of the 0·5 ml. graduation. The drain tube *m* is also a worth-while addition and consists simply of a short tube with a small bulb blown at one end. Immediately below the bulb a hole is blown in the stem of the tube so that when the bulb is pulled into the rubber, the hole is sealed off.

In fitting the upper (split) rubber disc, care should be taken to make the joints tight so that mercury and reagents accidentally spilled on top do not find their way into the jacket.

D. The Manometer. This should be made from thick-walled tubing of about 4 mm. bore and some 650 mm. long over the graduated portion. The graduations are best etched directly on the glass in 1 mm. divisions, but a mirror glass scale mounted behind the tube is almost as good. The zero mark should be arranged about 1 cm. below the 50 ml. graduation on the extraction chamber.

E. Rubber Tubing. The rubber tubing connecting the mercury reservoir with the rest of the apparatus should be good quality pressure tubing, about $\frac{1}{2}$ in. diameter with a bore of $\frac{1}{8}$ in. Synthetic tubing is definitely better than natural rubber in this application, since it is not so porous. The rubber joint at *n* is a length of heavier tubing, say $\frac{3}{4}$ in. diameter by $\frac{1}{8}$ in. bore. In Van Slyke's original apparatus, a mercury seal was fitted round this joint, but experience shows it to be unnecessary.

F. The Shaking Device. This usually consists of a small motor driving a simple crankshaft. In the interests of silence it should be well made and robust. The amplitude of oscillation should be about 2 in. at the top of the chamber and the speed should be some 300–400 revolutions per minute.

G. Reservoir Stations. Four positions of the mercury reservoir are required. Three are split retort rings fixed to the body of the apparatus at the positions shown, while the fourth may consist of a hook screwed into the bench about two feet below the bench-top.

Preparation of Apparatus for Use

Cleaning. Before taking a new apparatus into service the glass parts must be stripped and cleaned. Grease should be removed from the cocks by a solvent followed by wiping with *hardened* filter-paper or chamois-leather (ordinary filter-paper or cloth leaves fibres which spoil the gas-tightness of the cocks). Cleaning solution should then be drawn in, and after rinsing with water the tubes may be dried with acetone followed by a current of air.

Calibration. While the apparatus is still stripped, it is wise to check the calibration of the extraction chamber, upon which the accuracy of analysis depends. A convenient method is to attach a glass cock and jet (by means of pressure tubing) to the lower end of the extraction chamber, which should be supported vertically in a clamp. The chamber is filled with water by applying suction to the cup *c* (fig. VI.13) until it rises above the upper cock *b*. Water may then be delivered under oil by manipulating the lower cock and calibration effected as already described for burettes (p. 156). A reading glass should be used to observe the menisci during this process and the weighings should be accurate to 1 mg.

Assembly. The manometer, etc., may now be fixed to the wooden stand by means of screw clips with cork packing inserted between the tubes and

stand. Care should be taken to avoid straining the glass, but the attachment below the junction with the chamber should be firm.

After threading the lower rubber disc for the water-jacket on to the stem of the extraction chamber, the end of the stem and the end of the right-hand U-tube of the manometer assembly should be smeared lightly with rubber grease and the joint made glass to glass with the heavy pressure tubing. The water-jacket, thistle funnel, thermometer, etc., can be fitted and the clamp for the shaking device attached.

Next, the various cock keys should be greased and inserted in their barrels. The best method of greasing is as follows: *Filtered* petroleum jelly is smeared *lightly* over the key followed by a little *hard* rubber grease. The latter should be dabbed on at several points so that the petroleum jelly is not disturbed. The key is then warmed cautiously in an alcohol flame until the rubber grease runs into the soft paraffin. While still warm, the key is inserted in its barrel and rotated once or twice, *without excessive pressure*, until the whole cock presents a transparent appearance. Some experience is needed to judge the correct amount of grease for making a perfect seal but leaving the bore clean.

Filling. The apparatus is now ready to be filled with mercury. With all cocks fully open, mercury is poured into the reservoir, which should be held in the hand at about the level of the control cock *j*. When the mercury has reached the same level the reservoir should be lifted slowly so that some of the metal flows into the U-tubes, that in the reservoir being replenished as required. A certain amount of air will be entrained in the arms of the U-tubes, but is removed presently. Lifting of the reservoir is continued until mercury flows through the air-release cock *i* (fig. VI.12), which is then closed. Further raising of the reservoir fills the extraction chamber and after turning cock *b* so that a little mercury flows through the side-arm *o* (fig. VI.12), the latter is closed by a rubber teat. With *b* closed, the mercury is allowed to rise in the manometer until it passes through cock *f*, which is also closed.

The reservoir is now lowered well below the control cock *j* until the column breaks at the cross-joint. The space so produced will contain most of the entrained air at a low pressure. The reservoir is again raised slowly until the chamber and manometer are refilled with mercury. After placing the reservoir in the middle retort ring *2* air is ejected from the trap by opening cock *i* very cautiously. Any small amount of air collecting in the manometer can be neglected for the time being. Evacuation of the system and ejection of air from the trap should be repeated once or twice more.

It is now necessary to introduce a drying agent into the manometer tube as follows: With the control cock *j* open, the reservoir is raised above the level of the manometer cock *f*, which is then opened to expel air. About 0·5 ml. of *trimethylene glycol* is introduced into the open tube above the cock

and drawn into the manometer by lowering the mercury very cautiously. This is followed by a few millilitres of mercury to fill the bore of the cock, which is then closed. (*N.B.*—The cocks will not hold a vacuum unless their bores are filled with mercury.) At this stage the reservoir is lowered until the mercury in the manometer falls about 20 cm. The control cock *j* is then closed and the reservoir placed in the lowest ring. It is not desirable to lower the mercury further, since trimethylene glycol in the bottom part of the manometer may be a nuisance.

The water-jacket may now be filled. The most satisfactory solutions for this purpose are 15% glycerol or di-ethylene glycol in water, which entirely prevents the growth of moulds, etc. The jacket should be filled completely, but a small air-space must be left in the thistle funnel.

It only remains to eject the excess of trimethylene glycol from the manometer, and after resealing the cock with mercury, the apparatus is then ready for use.

Auxiliary Apparatus

Apart from the apparatus already described, a certain amount of ancillary equipment is required for all determinations; specialised accessories will be described on later pages.

(1) *Wash-bottle.* An aspirator or wide-mouthed bottle of about 1 l. capacity is supported on top of the wooden stand and is fitted with a rubber tube long enough to reach the top cup of the extraction chamber. The tube is furnished with a Mohr clip and a jet, preferably a stainless-steel serum needle with ground-off bevel. Glass may be used for the jet, but is very liable to be broken in the capillary below the cup, and if fragments of glass find their way into the two-way cock *b* the latter will be rendered useless.

(2) *Suction Line.* A second flexible tube fitted with clip and jet and attached to a source of suction is also required. If a vacuum line is used, two trap bottles should be put in circuit. The first (nearest the jet) need have a capacity of only 20 ml., and serves to collect any mercury carried over, while the second should be at least 1 l., since all spent solutions, etc., will be collected in it. In the absence of a vacuum line, a Winchester-quart bottle may be used as a suction reservoir and second trap combined. This may be evacuated from time to time by means of a filter pump. A single evacuation usually serves for at least ten analyses.

(3) *Mercury Bottle.* A narrow-necked bottle of about 50 ml. capacity and fitted with a cork through which a small hole ($\frac{1}{16}$ in.) has been pierced is convenient for containing mercury to be used in sealing cocks, etc.

(4) *Containers for Air-free Solutions.* Fig. VI.15 shows a suitable and robust pattern due to Sendroy.* The outer container is easily made by cutting

* Sendroy, J., *Ind. Eng. Chem.* (Anal. Edn.), 1937, **9**, 190.

the neck off a caustic-soda jar. When not in use, the tip of the jet should be kept immersed in a small tube of mercury to prevent ingress of air with any contraction of the contents.

(5) *Timer.* During analysis shaking is carried on for a definite time. A dark-room clock is probably the best indicator, but a calibrated sand-glass affixed to the body of the machine serves almost as well.

(6) *Modified Hempel Pipettes.* In some analyses gas is absorbed in special reagents outside the extraction chamber. For this purpose the modified Hempel pipette illustrated in fig. VI.16 is required.

It is absolutely essential that the bores of the cock *a* shall meet the capillary
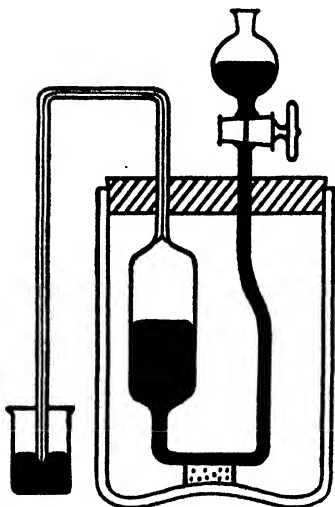
Fig. VI.15.
Container for Air-free Solutions.
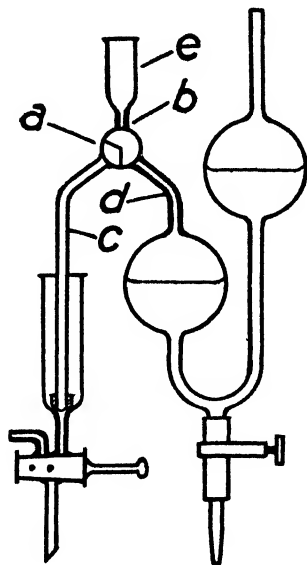
Fig. VI.16.
Modified Hempel Pipette.

tubes squarely. If the bore is chipped or countersunk, significant amounts of gas may become trapped and spoil the precision of the analysis.

These pipettes may also be used for containing air-free solutions, but are inferior to those of Sendroy (above) on account of fragility and of the liability of the cock to seize-up when used with alkaline solutions.

(7) *Pipettes.* Special pipettes (see Part III, pp. 143–147) of capacities 0·5 ml., 1·0 ml., 2 ml., 3 ml., and 5 ml. should be available. In some cases graduated straight pipettes controlled by the finger may be used.

(8) *Rubber Tips.* In order to transfer reagents or gas to and from the extraction chamber, it is necessary to fit pipettes, etc., with rubber adaptors. These are made from $\frac{1}{2}$ in. lengths of soft *natural* rubber tubing (synthetic

rubber is unsatisfactory in this application) by supporting the tube on a suitable metal rod and rotating the rubber against a grinding wheel until a hemispherical end is produced. The size of tubing to be used depends upon the outside diameter of the pipette tip. In general the wall thickness should be about ⅛ in. and the bore half that of the pipette diameter.

(9) *Enamel Trays.* Vessels containing mercury should be kept in trays so that in the event of breakage or spillage the mercury is confined and can be recovered.

(10) *Tap Grease.* Small quantities of *filtered* petroleum jelly and of hard rubber grease should be kept at hand in small closed pots.

(11) *Bamboo.* A piece of bamboo or similar cane capable of being split into narrow fibres is useful for removing obstructions from capillaries or bores of cocks. *On no account* should *wire* or other sharp material be used, since it produces minute scratches on the inside of the glass tubing which invariably lead to early failure of the parts.

General Technique

The following points of technique apply to most analyses performed with the manometric Van Slyke apparatus.

(1) *Reading Menisci.* The mercury meniscus in the manometer should be read to 0·1 or 0·2 mm. by using a lens and taking care to avoid parallax.

The bottom of the aqueous meniscus in the chamber must always be brought exactly to the mark, and again a lens should be used. A good light is required behind the extraction chamber. An opal bulb is suitable, but it must not be placed so close that the water-jacket is heated by radiation.

(2) *Adjustment of Gas Volume.* After the gases have been extracted during an analysis, the volume is reduced to 0·5 ml. or 2·0 ml. before the pressure is read. The reduction in volume must take place fairly rapidly yet without undue agitation of the liquid surface, in order that re-solution of gas is minimal. This applies particularly when carbon dioxide is present, since on account of its high solubility it is necessary to stop the admission of mercury to the chamber *immediately* the meniscus reaches the mark. By manipulation of the control cock j (fig. VI.12) the mercury is allowed to rise smoothly until the shoulder of the main bulb is reached. The cock is then closed gradually until the rate of travel of the meniscus is sufficiently slow to allow it to be stopped precisely at the mark.

If "overshooting" occurs, the chamber must be re-evacuated and shaken for one minute, after which the volume may be reduced again.

If only gases of low solubility such as oxygen, nitrogen, or carbon monoxide are present, the re-absorption is so little that the meniscus can be re-adjusted slightly without further evacuation and shaking.

(3) *Testing for Leaks.* Before making a series of analyses it is advisable to check the apparatus for gas-tightness, as follows:

With the reservoir at station 2 (fig. VI.12) and the chamber full of mercury, 5 ml. of water are placed in the cup, the cock below the cup being left open, but the control cock closed. The reservoir is lowered and the control cock opened slowly to admit about 3 ml. of water to the chamber. 0·5 ml. of mercury is placed in the cup and a little drawn through the bore of the upper cock, which is then closed.

With the control cock open, the reservoir is lowered until the mercury reaches the 50 ml. mark, when the control cock is closed and the reservoir returned to station 3. The evacuated chamber is then shaken for 3 minutes, after which time the gas volume is reduced to 0·5 ml. and the manometer is read.

The evacuation and shaking should be repeated twice more, any difference in the readings indicating a leak.

If the readings decrease successively, then the cock at the top of the manometer should be inspected and regreased. An increasing reading usually means that the cock above the chamber is leaky. In this event the key should be removed, cleaned, regreased, and the bores sealed with mercury. The test should be applied again and if the readings still increase then the flexible joint between the extraction chamber and the rest of the apparatus should be renewed. Leakage at this point is, however, rare.

(4) *Lubrication of Cocks.* All cocks should be kept gas-tight and free-working. Any tendency to bind must be corrected *immediately* by relubrication according to the following procedure:

(a) *Upper Cock b* (fig. VI.12). The cap is removed from the side-arm and the mercury seal drawn into the chamber. The cock is then turned through 180° and about 10 ml. of water drawn in, together with a little air. After closing the control cock and placing the reservoir at station 3 the upper cock is removed and greased as described on p. 539.

With the freshly greased cock turned to put cup and chamber in communication the reservoir is raised and the control cock opened fully so that the water is forced through the capillary under enough pressure to dislodge any plug of grease partly obstructing the bore. If it fails to do so, more water should be introduced into the chamber and its level arranged so that it just appears in the capillary below the cup. About 1 ml. of concentrated sulphuric acid is then drawn in the chamber above the water and immediately forced out again. The heat of reaction when the acid and water mix below the cock is sufficient to melt the offending plug, which then floats out.

All that remains is to seal the side capillary with mercury, as already described (p. 539).

(b) *Control Cock j and Trap Cock i* (Fig. VI.12). The reservoir is raised until mercury passes into the cup above the chamber. The two-way upper

cock is closed and the reservoir supported a little below station 3 with the control cock open. The trap cock i is then opened so that the mercury falls below the control cock.

Both this and cock i may be removed and regreased, after which, the reservoir is raised until the mercury just passes through the trap cock.

(c) *Manometer Cock f* (fig. VI.12). With the reservoir at *2* and the chamber full of mercury, the key may be removed and regreased. After greasing, fresh tri-methylene glycol should be introduced and the bore sealed as described on p. 539.

Removal of Air from Trap

From time to time it is necessary to remove air collecting above the control cock. This is effected by arranging the apparatus as in (c) above, and opening the trap cock *very cautiously*.

Cleaning the Extraction Chamber

The apparatus should be kept clean. Foreign material between the cock and 2 ml. graduation introduces a considerable error into the analysis, and so it is advisable to make the thorough cleaning of the extraction chamber a matter of routine at the end of each estimation.

Contamination with grease is avoided by the lubrication procedure already described.

Protein precipitated in blood analyses can be removed as follows: After the analysis, the spent solution is ejected from the chamber, sucked off, and the cock closed. The reservoir is lowered until the mercury falls two-thirds of the way down the chamber. At this point the *control cock is closed* and the cup filled with $0\cdot1N$ caustic soda containing a little sodium hydrosulphite. This latter is introduced into the vacuous chamber by opening the upper cock in short "bursts," so scrubbing the walls effectively.

Alternatively a 40% solution of urea is often effective. The addition of a "wetting-out" agent to the urea solution is a decided improvement. Finally, the apparatus should be washed with water and then with $0\cdot1N$ lactic acid.

When not in use, the chamber should be left full of water and the reservoir at station *3* (fig. VI.12). On no account should the instrument be left for long periods with the reservoir at station *1* or *2*, since the pressure of mercury may force the control cock key out, with unfortunate results! Also, the chamber must not be left standing full of mercury with all cocks closed. A rise of temperature may expand the mercury sufficiently to fracture the glass.

Preparation of Air-free Solutions

About 30 ml. of the solution are introduced into the extraction chamber, the cock sealed with mercury, and the chamber evacuated.

After shaking for 3 minutes, the extracted air is ejected from the chamber and the process repeated twice more.

The air-free solution is transferred to a container (fig. VI.15) as follows: The capillary of the container is filled with mercury and fitted with a rubber adaptor (p. 541). The mercury reservoir of the Van Slyke apparatus is raised to station *1* (fig. VI.12) and a few millilitres of air-free solution allowed to enter the cup. The tip of the container is placed under the solution and about 1 ml. of mercury allowed to run through the jet, which is then pressed firmly in contact with the base of the cup, the tap on the container being left open. Cock *b* is next opened fully and the solution fed into the container by manipulating the control cock *j*. As soon as a little mercury has entered the jet from the extraction chamber, the control cock is closed, followed by cock *b* and finally that on the container. The container can then be put on one side with its jet immersed in mercury, whilst the reservoir must be removed immediately to a lower level and the chamber washed out thoroughly.

Calculations

The majority of methods published for use with the Van Slyke apparatus include tables of factors (e.g. Tables I, II, III of the Appendix, pp. 579–581) by which the observed partial pressure of a given gas is multiplied to give the actual concentration of the gas.

Direct calculation, though seldom necessary, can, however, be made by using the theoretical equation given by Van Slyke and Neill,[*] i.e.

$$(1) \qquad V_{(0°\ 760\ mm.)} = P \times \frac{i.a}{760(1+0\cdot00384t)} \times \left(1+\frac{S\alpha'}{A-S}\right)$$

where in the *1st Term*:

P is the observed partial pressure obtained from the difference in manometer reading before and after removal of the gas.

2nd Term

i is an empirical factor taking account of the gas re-absorbed during the time the extracted gases are being reduced to standard volume.

For slightly soluble gases, such as O_2, N_2, CO, $i=1\cdot0$, but for CO_2 $i=1\cdot017$ at $a=2\cdot0$ ml. and $1\cdot037$ at $a=0\cdot5$ ml.

a is the volume at which P is measured, usually $2\cdot0$ or $0\cdot5$ ml. The division in the second term converts V.P. to N.T.P., but the temperature coefficient $\left(\dfrac{1}{1+0\cdot00384t}\right)$ is not identical with $\dfrac{273}{273+t°}$, since it also includes a mercury-in-glass expansion factor.[*]

[*] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

3rd Term

S is the volume of aqueous solution in the chamber after extraction.

A is the total volume of the chamber, viz. 50 ml.

α' is the distribution ratio (α) for the particular gas multiplied by the temperature coefficient.

It will be seen that since P is a *difference* of pressure, the effect of barometric pressure and the aqueous vapour tension cancel out and do not appear in the equation.

It is usually more convenient to record the concentration of gas either as volumes per 100 volumes of sample or as millimoles per litre of sample, in which case the equation becomes:

(2) Volume per cent. of any gas in sample

$$= P \times \frac{0 \cdot 1316 \; i.a}{\text{(ml. sample)}} \times \frac{1}{(1 + 0 \cdot 00384t)} \times \left(1 + \frac{S\alpha'}{A - S} \right)$$

$$= P \times volume \; per \; cent. \; factor.$$

(3) Millimoles of gas per litre of sample

$$= P \times \frac{0 \cdot 0587 \; i.a}{\text{(ml. sample)}} \times \frac{1}{(1 + 0 \cdot 00384t)} \times \left(1 + \frac{S\alpha'}{A - S} \right)$$

$$= P \times millimoles \; per \; litre \; factor.$$

Equation (3) needs modification in the case of carbon dioxide since 1 mg. molecule occupies only 22·26 ml. at 0° and 760 mm. instead of 22·40 ml.* The factor 0·0587 then becomes 0·0591.

Published tables are calculated *with* values of *a* exactly *equal to* 2·0 ml. or 0·5 ml. If, on calibration, a particular apparatus has values of *a* sensibly different from 2·0 or 0·5 ml., either new tables incorporating the observed value of *a* should be prepared or the results obtained by use of the standard tables multiplied by a suitable correction factor. Viz. if a chamber has an *a* value of 0·49 ml. instead of 0·5 ml., the result must be multiplied by $\frac{49}{50} = 0·98$.

REPRESENTATIVE ANALYTICAL PROCEDURES

Example 1: Determination of Carbon Dioxide in Blood, Plasma, or Aqueous Carbonate Solutions.†

METHOD FOR 1 ML. SAMPLES.

REAGENTS.

1. *Lactic acid, approximately* 0·1N ; *carbon dioxide-free.* Dilute 1 volume of lactic acid of sp. gr. 1·20 to 100 volumes with water and aerate or boil to remove carbon dioxide.

* Guye, P. A., and Pintza, A., *Mem. Soc. Phys. et Hist. nat. Geneve*, 1908, **35**, 551.
† Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

2. *Sodium hydroxide, approximately* 1N, *air-free*. Cover solid sodium hydroxide, contained in a paraffined bottle, with an equal weight of water and allow to stand until saturated. Any carbonate settles out, leaving the supernatant liquid about 18N.

Dilute 5·5 ml. of this solution to 100 ml. to give an approximately 1·0N solution, and then render it air-free by the procedure given on pp. 544–545.

3. *Capryl alcohol or Tri-butyl citrate*. These are used as anti-foams, and should be contained in dropping bottles.

PROCEDURE. The extraction chamber is filled with mercury and 1 drop of anti-foam is drawn into the capillary below the cup. 2·5 ml. of 0·1N lactic acid are placed in the cup and a 1 ml. stop-cock pipette (Section III, p. 145, fig. III.3, a) filled with the sample and fitted with a rubber tip is placed in contact with the bottom of the cup. Before filling the pipette, it is wise to add a drop of caprylic alcohol to the sample in order to avoid air-bubbles being trapped in the stem.

With the reservoir at station *3* (fig. VI.12) both cock b and the pipette cock are opened. Cautious opening of the control cock draws the sample into the chamber, the *pipette cock* being closed when the meniscus reaches the lower mark. The pipette is lifted slightly and the 2·5 ml. of lactic acid follows the sample into the chamber, making a total of 3·5 ml. The control cock is closed *as soon as the lactic acid just fills the capillary* below cup c. About 1 ml. of water or dilute lactic acid is placed in the cup and followed by a little mercury. Any air bubbles under the mercury must be dislodged with a fibre.

Enough *mercury* is drawn through cock b to seal the bore and the cock is closed. The chamber is then evacuated and shaken for 2 minutes.

After extraction, the volume is reduced to 2·0 ml. (see p. 542) and the manometer is read (=P_1).

As soon as the reading is made the reservoir must be lowered with the control cock open until the gas space is expanded from 2 ml. to between 5 ml. and 10 ml. The control cock is closed and 2 ml. of air-free sodium hydroxide introduced into the cup. 1 ml. of this solution is drawn into the chamber during about 30 seconds. Caution is required here, since careless opening of cock b will draw the whole 2 ml. of solution and a great deal of air into the chamber.

It is necessary to use 2 ml. of solution so that the top (unused) 1 ml. guards the lower layer against absorption of atmospheric carbon dioxide.

After the carbon dioxide is absorbed, a little mercury is drawn through the cock to clear any sodium hydroxide in the 0·5 ml. portion of the chamber and to seal the bore.

The volume is again reduced to 2 ml. and a second reading (P_2) made.

After noting the temperature, the solutions may be ejected from the chamber.

Then $P_{[CO_2]} = P_1 - P_2 - C$ at $t°$ C.

The "*C*" *correction* is determined by extracting 3·5 ml. of 0·1N lactic acid for two minutes and repeating the absorption as in an analysis. Then $C = P_1 - P_2$. It is made up of two parts, (1) the difference in manometer level due to the hydrostatic pressure of the 1 ml. of sodium hydroxide added, and (2) any residual gas introduced by the reagent, and should not vary in successive analyses.

The $P_{[CO_2]}$ so obtained is multiplied by the appropriate factor as given in Table I, p. 579, to give volumes per cent. or millimoles per litre of carbon dioxide.

Example 2: Precise Determination of Carbon Monoxide in Blood.*

One or two volumes per cent. of carbon monoxide in blood can be determined with an accuracy of 0·02%. Blood samples of between 1 and 5 ml. may be used, though a special correction factor is required for a 5 ml. sample.†

Sendroy‡ has applied the above method to the determination of carbon monoxide in air. Concentrations between 1 in 2,000 and 1 in 350 parts by volume can be determined with an error of less than 5%. Although not quite so accurate as the iodine pentoxide method, the Sendroy technique is very valuable for occasional analyses and does not require the somewhat elaborate one-purpose gas train.

PRINCIPLES. The blood is introduced into the extraction chamber together with an acid ferricyanide reagent. All gases, including carbon monoxide, are extracted and transferred to a Hempel pipette, where carbon dioxide and oxygen are absorbed. The carbon monoxide and nitrogen remaining are returned to the extraction chamber, and the carbon monoxide is determined by absorption with cuprous chloride.

REAGENTS.

1. *Acid ferricyanide* (Rappaport's reagent).§ Potassium ferricyanide, 4·0 g.; urea, 400·0 g.; saponin, 3·0 g.; capryl alcohol, 5·0 ml.; water to 1 l.

2. *Winkler's cuprous chloride solution.* Place 200 g. of pure cuprous chloride, 250 g. of ammonium chloride, and 750 ml. of water in a bottle just large enough to contain them. Close at once with a rubber stopper, and after shaking to dissolve the salts place a coil of copper wire in the solution; re-stopper. On standing any oxy-chloride settles out, leaving a clear supernatant solution.

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* Sendroy, J., and Liu, S. H., *J. Biol. Chem.*, 1930, **89**, 133; see also Peters and Van Slyke, "Quantitative Clinical Chemistry," Vol. II (London, 1932).

† Published in paper by Van Slyke, D. D., and Robbins, R., *J. Biol. Chem.*, 1927, **72**, 39.

‡ Sendroy, J., *J. Biol. Chem.*, 1932, **95**, 599.

§ This very stable reagent is a great improvement on the one used in other methods, since blood protein largely remains in solution. Hence the chamber can be cleaned much more easily between analyses.

3. *Alkaline pyrogallol.* Make a caustic potash solution of sp. gr. not less than 1·55 (300 g. caustic potash stick, 200 ml. water). Add 15 g. of pyrogallol to 100 ml. of this caustic potash solution and store in a small aspirator with paraffined rubber fittings. Portions are withdrawn into the modified Hempel pipette (fig. VI.16) as required.

The solution improves on keeping.

4. *Sodium hydroxide solution, air-free* (see p. 544).

5. *Lactic acid.* 1·0N.

AUXILIARY APPARATUS. A modified Hempel pipette (fig. VI.16), filled with alkaline pyrogallol, and with the contents protected from the atmosphere by a layer of paraffin.

PROCEDURE USING 2 ML. SAMPLE.

(1) *Extraction of Gases from Sample.* Two drops of "anti-foam" are drawn into the capillary below the cup *b* (fig. VI.12) and 8 ml. of Rappaport's reagent together with 1 ml. of 1N lactic acid are placed in the cup. The acid ferricyanide reagent is introduced into the chamber, which is evacuated and shaken for 2 minutes after sealing the cock. The extracted gases are ejected and the extraction is repeated.

After the second extraction, 5 ml. of the now air-free reagent are run into the cup, 2 ml. of blood are introduced into the chamber, followed by · 1 ml. of the air-free ferricyanide solution, making a total of 7 ml. in the chamber. The mixture is extracted for 3 minutes and the gas space reduced to between 5 and 10 ml. Any carbon dioxide present is absorbed with 1 ml. of air-free sodium hydroxide, as described on p. 547. The meniscus is brought exactly to the 2 ml. mark and P_1 is obtained.

(2) *Transfer of Gases to the Hempel Pipette.* During the time the blood gases are being extracted, the Hempel pipette containing the pyrogallate is prepared as follows: The cock *a* (fig. VI.16) is opened so that a little solution and any bubbles of gas are ejected from the capillary *b*. One drop of caprylic alcohol is placed in the capillary and a few millilitres of mercury in the cup. The cock is then turned to fill limbs *c* and *d* with mercury. Finally, a rubber tip is fitted to the jet.

Immediately the P_1 reading has been made, the reservoir of the apparatus is held in the hand so that the mercury level is about 1 cm. above the level in the chamber. The control cock is opened so that the gas volume is reduced from 2 ml. and the pressure is increased to slightly above atmospheric. The control cock is closed again and the reservoir placed at station *2* (fig. VI.12).

1 or 2 ml. of mercury are placed in cup *c* (fig. VI.12), care being taken to remove every trace of gas in the capillary below it. The rubber-tipped jet of the pipette is placed just below the mercury surface and its cock *a* (fig. VI.16) is opened so that a little mercury flows from the tip. With

cock a still open, the pipette is held firmly in the base of the cup c (fig. VI.12) and its cock turned through 120° so that the limbs c and d (fig. VI.16) are connected.

At this point the two-way cock b (fig. VI.12) is opened. A little gas should pass from the chamber into limb c (fig. VI.16) of the pipette. The control cock j (fig. VI.12) is now opened cautiously so that the remainder of the gas passes through cock b (fig. VI.12) followed by the smallest possible amount of blood solution.

After closing the control cock, the pipette is removed and limb c (fig. VI.16) is freed from blood solution by mercury from the cup e (fig. VI.16). The trace of blood and the column of gas in limb d is forced into the pyrogallate by further rotation of cock a. The pipette is "swirled" slightly and then set aside for three or four minutes to complete absorption of oxygen.

During this time the extraction chamber is washed free from blood solution (see p. 544). When clean, 9 ml. of water are introduced and de-gassed as described for the blood-ferricyanide mixture. After ejection of gas, 2 ml. of the water are run into the cup, leaving 7 ml. in the chamber.

(3) *Transfer of Carbon Monoxide and Nitrogen from Pipette to Chamber.* 1 or 2 ml. of mercury are placed in the cup above the chamber of the manometric apparatus and the pipette is placed in the base of the cup while mercury is issuing from the jet. Cock a (fig. VI.16) is again turned to put the bulb into connection with the limb c and with the extraction chamber of the Van Slyke apparatus. Immediately pyrogallate appears in limb c of the Hempel pipette, the 120° cock must be turned so that mercury from the cup e (fig. VI.16) follows the gas bubble through the jet into the chamber.

When mercury has passed through cock b (fig. VI.12), the latter is closed, the pipette is put aside, the carbon monoxide/nitrogen mixture in the chamber is expanded to 2·0 ml., and the manometer read (P_2). Then

$$P_{[O_2]}=P_1-P_2-C_{[O_2]}.$$

The gases are then reduced to 0·5 ml. volume, at which another reading (P_3) is made.

Absorption of Carbon Monoxide

6 ml. of Winkler's solution are placed in the cup of the manometric apparatus. With the reservoir at 3 (fig. VI.12) to provide a slight negative pressure and control cock open, 5 ml. of the solution are admitted to the chamber over a period of 2 minutes. The absorption of carbon monoxide is complete if the negative pressure is no greater than that stipulated.

The nitrogen remaining is expanded to 0·5 ml. volume and P_4 is noted. Then

$$P_{[CO]}=P_3-P_4-C_{[CO]}.$$

The "C" Corrections

The "$C_{[O_2]}$" is determined by blank analysis for P_2 using 7 ml. of air-free ferricyanide solution instead of 5 ml. of solution+2 ml. of blood.   Then

$$C_{[O_2]}=P'_1-P'_2.$$

The "$C_{[CO]}$" is determined by extracting 5 ml. of water for 1 minute and ejecting the gases.   The level is lowered to 0·5 ml. and the manometer is read (=P'_3).   5 ml. of Winkler's solution are admitted and after adjustment to 0·5 ml., P'_4 is noted.   Then

$$P_{[CO]}=P'_2-P'_3.$$

CALCULATIONS.

The composition of the sample can be determined by means of the factors given in Table II of the Appendix, p. 580.

Example 3: Determination of Carbon by Wet Combustion.*

By this method carbon can be determined accurately and very speedily in non-volatile compounds.   In the micro- and sub-micro-analyses amounts of carbon between 2 and 3·5 mg. and 0·3 and 0·7 mg. respectively are determined, the corresponding mean errors being only 0·2% and 0·5%. After weighing the sample, analyses can be completed in 15–20 minutes. Wet sludges (blood, pus, faeces, etc.) and solutions can easily be analysed, and the method has the further advantage that nitrogen, sulphur, phosphorus, halogen, etc., do not interfere.

The same method has been applied by Hoagland† to the micro-determinations of sulphate, phosphate, and magnesium.   The sulphate and phosphate are determined by combustion of their benzidine salts, and magnesium by combustion of its 8-hydroxyquinoline complex.   Further applications of the same principle can be envisaged.

PRINCIPLES.   The carbon compound is digested for 1–3 minutes at reduced pressure in an oxidising mixture.   Carbon dioxide is evolved quantitatively and absorbed in alkali contained in the extraction chamber.   After ejection of unabsorbed oxygen and nitrogen, the alkali is neutralised and the pressure of the liberated carbon dioxide is measured.

APPARATUS.

1. Van Slyke-Neill manometric apparatus (fig. VI.12) with graduations at 0·5 ml., 2·0 ml., and 10 ml. (see fig. VI.13).

2. Combustion vessel and connecting tube (fig. VI.17).

3. Container for carbon dioxide-free alkali (fig. VI.18).

4. Protected bottle for oxidising mixture (fig. VI.19).

* Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, 1940, **136**, 509.
† Hoagland, C. L., *J. Biol. Chem.*, 1940, **136**, 543, 553.
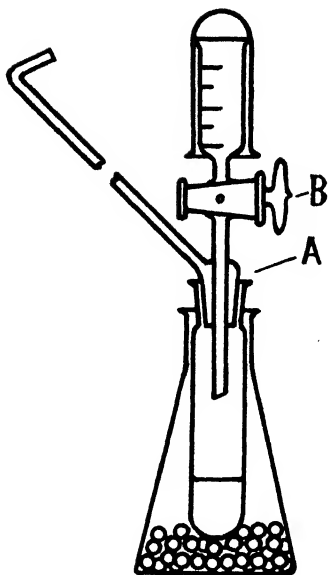
Fig. VI.17. Combustion Vessel and Connecting Tube for Use in the Determination of Carbon.

5. Dust-proof storage vessel for combustion tubes.

6. Aluminium scoops, with counterpoise for weighing and transfer of sample (fig. VI.20).

7. Calibrated glass spoons.

8. Coarse silver sand, cleaned by boiling in "combustion mixture."

REAGENTS.

1. *Combustion mixture.* Mix 25 g. of chromium trioxide, 167 ml. of phosphoric acid (d. 1·7), and 333 ml. of fuming sulphuric acid (20% SO_3) in a litre glass-stoppered flask and heat with gentle agitation to oxidise impurities. When the temperature has risen to 150° C., cover the open neck with a beaker and allow the flask to cool. After cooling, insert the stopper and use the beaker as a dust cover.

For use in analyses, transfer small portions to the bottle illustrated in fig. VI.19.

2. *Potassium iodate, A.R., powdered.*
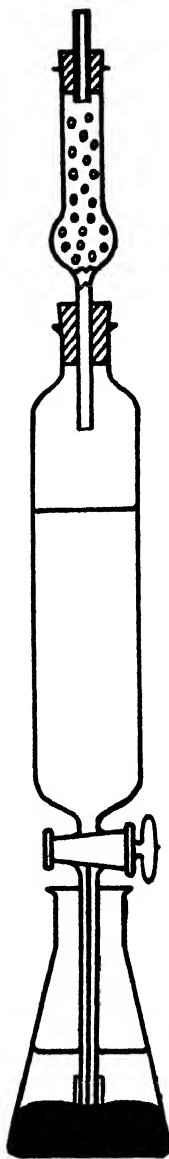
Fig. VI.18. Container for Carbon Dioxide-free Solutions.

3. *Sodium hydroxide*, 0·1N *carbon dioxide-free, containing* 0·3M *hydrazine.*
Dilute 18N sodium hydroxide (p. 547) with carbon dioxide-free water until,
after titrating, the strength is 0·815±0·005N, and store in a waxed aspirator
fitted with a soda-lime guard tube and capillary outlet.

Weigh into a 500 ml. volumetric flask 5·0 g. of hydrazine sulphate and
fill to the mark with 0·815N sodium hydroxide, taking care to prevent
access of carbon dioxide. After dissolving the salt,
transfer the solution to vessel (fig. VI.18, p. 552) by
suction, leaving the last 50 ml. in the flask. This solu-
tion must be changed after a month, since the hydrazine,
necessary to absorb free halogens, decomposes slowly.

4. *Lactic acid, 2N.*

5. *Sodium hydroxide, approximately 5N*, contained in
a vessel similar to that used for solution 3.

PROCEDURE ("MICRO" ANALYSIS).

1. (a) *Dry Substances.* An amount of substance,
containing between 2·5 and 3·5 mg. of carbon, is placed
in the end of a scoop (fig. VI.20) and weighed with the
usual precautions (pp. 14–17). With the scoop held in
bone-tipped forceps, the sample is introduced into the
bottom of the combustion tube (fig. VI.17). The scoop
is then removed and reweighed. Alternatively, standard combustion boats
can be used. The tube and contents must then be stored under a dust-
proof cover.

Fig. VI.19. Protected
Reagent Bottle—for
Storage of Oxidation
Mixture.

(b) *Aqueous Solutions.* The amount of solution should not exceed 2 ml.
and should be delivered to the bottom of the combustion tube. After
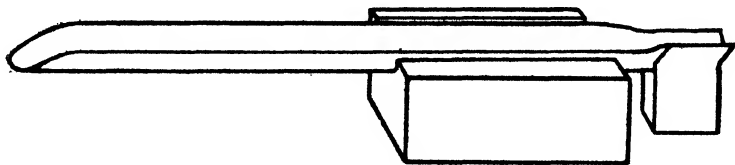
Fig. VI.20. Aluminium Weighing Scoop.

introduction of the solution, the tube is placed in a vacuum desiccator
until evaporation is complete.

(c) *Non-aqueous Solutions.* The solvent is best removed by heat and
reduced pressure with the most stringent precautions to avoid contamination
by dust. Because of the ease with which dry films are oxidised, the com-
bustion should be carried out as soon as possible after evaporation.

2. 200 mg. of potassium iodate are introduced with a calibrated glass
spoon into the combustion tube, together with a little sand. The stopper

and connecting tube (fig. VI.17) are fitted, the standard joint and cock *a, b* being sealed with phosphoric acid. Next, the end of the connecting tube is fitted at *o* (fig. VI.12) to the Van Slyke-Neill chamber, which should be full of mercury. Finally, 2 ml. of combustion mixture are placed in the cup of the combustion tube and the latter is covered.

3. Cock *b* (fig. VI.12) is opened to the combustion tube and the mercury is lowered to evacuate the system. The cock is then closed and the mercury is raised until the trapped air is under slight positive pressure. Cock *b* is opened *to the cup c* and the air is ejected. This removal of air eliminates any blank due to atmospheric carbon dioxide and assists subsequent transfer of evolved gas from the combustion tube to the extraction chamber.

4. The alkali-hydrazine container (fig. VI.18) is fitted with a rubber tip and the jet placed in the cup *c* of fig. VI.12 under a little mercury. Cocks *b* and *j* are opened and the mercury is lowered slowly by manipulating the control cock until the mercury/alkali junction is about 1 mm. above the 2 ml. mark on the chamber. The container cock is closed and the vessel removed. A few drops of mercury are then drawn through the capillary of cock *b*, which is closed. The chamber now contains exactly 2 ml. of alkali solution. The cup *c* must be washed out at once with $0.1N$ lactic acid, otherwise traces of carbonate form and spoil subsequent analyses.

5. The mercury reservoir is now lowered until the manometer falls to about the level of the 2 ml. mark on the chamber. The control cock is closed and the reservoir placed at station *3*. The cock *b* is left open to the combustion tube during this adjustment.

6. 2 ml. of combustion mixture are admitted from cup *B* (fig. VI.17) of the connecting assembly, care being taken to avoid admitting air. The tube is then heated over a micro-flame to boiling, the rate of boiling being adjusted so that the foam is not more than half-way up the tube. Carbon dioxide and oxygen are evolved and tend to depress the mercury level in the extraction chamber. To compensate, mercury is admitted from the reservoir as often as is required to maintain the gas space at about 1 ml. The manometer level rises during this time and reaches the top after a minute or so. Control cock *j* (fig. VI.12) is then left fully open. Vigorous boiling continues at about 600 mm. pressure and is maintained for $1\frac{1}{2}$ minutes.*

Boiling should not be continued for longer than 2 minutes, since excessive production of oxygen and iodine would then occur. During the last half-minute's boiling, the foam should be allowed to reach the upper part of the tube so that any adhering material present is oxidised.

7. Then the flame is reduced somewhat and the mercury reservoir is raised and lowered 20–25 times with the control cock open. Each time the

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* In the original paper, this time is claimed to be adequate for all compounds. Experience shows that certain compounds, notably phosphates, require 2 minutes.

mercury should be lowered as far as the 50 ml. mark and raised until about 5 ml. of gas space remains.

The fluid boils at each reduction of pressure and all carbon dioxide is removed and absorbed. At the end of absorption the two-way cock b (fig. VI.12) is closed and the combustion tube, etc., are removed.

8. The capillary outlet o (fig. VI.12) is connected with the mercury bottle illustrated in fig. VI.21 and sufficient mercury is drawn through to seal the cock.

The control cock is opened and the reservoir is raised until the level of mercury in it is about 1 cm. above cock b (fig. VI.12). The control cock is closed and cock b is opened. The control cock is then opened slowly so that the unabsorbed gases are ejected from the chamber. When the alkali reaches the bore of the cock b, further admission of mercury is stopped and cocks j and b are closed. Finally, a little mercury is drawn into the chamber to seal the cock. The bubble of carbon dioxide-free air from the bore is also admitted, but this is of no consequence.

9. 1 ml. of $2N$ lactic acid is admitted to the chamber from a stop-cock pipette (p. 145, Section III). After sealing and closing the cock, the mercury is lowered to the 50 ml. mark and shaken for 30 seconds. As carbon dioxide is evolved, mercury is re-admitted to keep the level at 50 ml. The chamber is shaken for a further $1\frac{1}{2}$ minutes to complete extraction. At the end of this time mercury is re-admitted with due precautions (p. 547) until the gas volume is 10 ml. A manometer reading $=P_1$ is then taken.
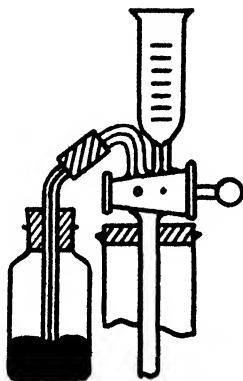
Fig. VI.21.
Mercury Vessel for Sealing Cock of Van Slyke Chamber.

10. The reservoir is placed at station 3 (fig. VI.12) so that the gas is slightly expanded, and 0·5 ml. of $5N$ sodium hydroxide is admitted to the chamber. Caution is required to avoid admitting air. The carbon dioxide is completely reabsorbed in about 30 seconds, the cup is then washed free of alkali with 0·1N lactic acid and the cock is resealed, sufficient mercury being allowed into the chamber to remove any alkali trapped in the 0·5 ml. portion of the chamber.

The solution is raised and lowered between the 10 ml. and 0·5 ml. marks to effect mixing and then its level is arranged just below the 10 ml. mark until drainage is complete. Finally, it is adjusted exactly to 10 ml. and a second reading $=P_2$ obtained. Then

$$P_{[CO_2]}=P_1-P_2-C.$$

The "C" correction is obtained by blank analysis and need only be determined once for each series of combustions.

CALCULATIONS.

Reference to Table III, p. 581, gives the factor by which $P_{[CO_2]}$ must be multiplied to find milligrams of carbon in the sample. To obtain these factors the formula on p. 545 is multiplied by the atomic weight of carbon ($=12\cdot01$). Thus:

$$Factor = \frac{0\cdot0007099\,i.a}{1+0\cdot00384t} \left(1 + \frac{S\alpha'}{A-S}\right)$$

Sub-micro Analysis.

The technique is as above except that:

  (i) Only 100 mg. of potassium iodate are used.

  (ii) Only 1·5 ml. of combustion mixture are used.

  (iii) Pressures are read at 2 ml. volume.

Example 4: Determination of Free α-Amino-acids with Ninhydrin.*

PRINCIPLES. When boiled in water with an excess of ninhydrin at a *p*H between 1 and 5, carbon dioxide is evolved quantitatively from amino-acids of two types:

R—CH—COOH   R—CH—COOH
| and | (e.g. proline and hydroxy-proline)
NH₂   NH—CH₂R'
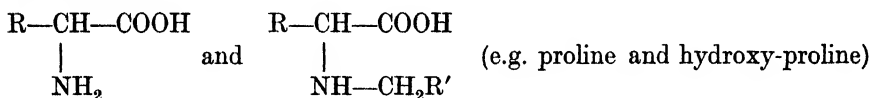
α-Ketonic acids (e.g. pyruvic acid) react similarly, but decompose on boiling in absence of ninhydrin, and so can be removed. Peptides, simple carboxylic acids, hydroxy-acids, amines, and amides are generally inert. So too are amino-acids in which the amino group is di-substituted or in which the carboxyl group has been esterified.

The carbon dioxide produced in this reaction is transferred quantitatively to the Van Slyke apparatus, absorbed in alkali, regenerated by acidification, and its pressure is then measured.

When used in conjunction with the gasometric estimation of amino groups (example 5, p. 558), this procedure can be adopted for the estimation of certain amino-acids in mixtures, such as protein hydrolysates.†

APPARATUS.

1. *Van Slyke-Neill manometric apparatus.*

2. *Storage vessel for a 0·5N sodium hydroxide* (fig. VI.17).

3. *Reaction vessels.* The glass-jointed pattern *b* is preferable, although *a* is cheaper (fig. VI.22). If *a* is used the minimum amount of rubber must be exposed to the gas, and a special rubber of low sulphur content is required.

4. *Glass spoons*, calibrated to contain 50 and 100 mg. of reagents.

* Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, 1941, **141**, 627.

† Van Slyke, D. D., *J. Biol. Chem.*, 1929, **83**, 425.

5. *Water-bath.* This should be of metal and of relatively large volume. A heater sufficiently large to keep the water boiling even when the cold reaction vessels are introduced is necessary.

6. *Weights*, to sink reaction vessels in the water-bath.

REAGENTS.

1. *Ninhydrin* (triketo-hydrindene hydrate).*

2. *Buffer solutions.* The following may all be required, though for normal purposes the one giving pH 2·5 suffices:

For pH 4·7: Tri-sodium citrate 17·65 g. plus citric acid, 8·40 g.

For pH 2·5:   ,,   ,,   2·06 g.   ,,   ,,   19·15 g.

For pH 1·0: 1·0M phosphoric acid (0·2 ml. of 6M acid plus 1 ml. of water). The citrate buffers are powdered, mixed to a cake, and then are repowdered and used dry.

3. 0·5N *sodium hydroxide, carbon dioxide-free.* See p. 547.

4. 5N *sodium hydroxide.* See p. 547.

5. *Lactic acid*, 2N.

PROCEDURE. In this analysis the "sub-micro" range corresponds to an amount of substance containing 0·035–0·18 mg. of carboxyl carbon and the "micro" range to 0·7 mg. In the first case pressures are measured at 0·5 ml. and in the second case at 2·0 ml. volumes.

"*Micro*" *Scale Analysis.* 4–8 mg. of dry sample are weighed in a scoop, transferred to a reaction vessel, and dissolved in between 1 and 5 ml. of water. Alternatively, a corresponding volume of solution may be taken.

According to the pH required, 50 mg. of buffer are added for volumes

Fig. VI.22. Reaction Vessels for Amino-acid Estimations.

of 1 or 2 ml. or 100 mg. for volumes of 3–5 ml. For work at pH 1, 1 ml. of M phosphoric acid is added per millilitre of sample.

Carbon dioxide is removed from the solution and keto-acids, etc., are destroyed by boiling for not less than 1 minute, silver sand being added to promote smooth boiling and capryl alcohol to prevent foaming.

After boiling, the vessel is stoppered to prevent re-absorption of carbon

* For preparation see Teeters, W. O., and Schriner, R. L., *J. Amer. Chem. Soc.*, 1933, **55**, 3026.

dioxide, cooled below 20° C. and 50–100 mg. of ninhydrin are added from a calibrated spoon. The reaction tube is evacuated as rapidly as possible to about 1 mm. pressure *immediately* after the addition of the ninhydrin, and must be closed as soon as the required pressure is reached. The preliminary cooling is essential in order to quench the reaction during the evacuation period, and much greater latitude in operating time is obtainable by cooling to below 15° or, if possible, to below 10°.

The reaction vessels are then immersed *completely* in a *vigorously boiling* water-bath for from 5–30 minutes, depending upon the volume of the reactants, the amount of ninhydrin, and the pH (see fig. A.1 on p. 582 for details). The vessels should be shaken after the first minute, and the recommended boiling time adhered to strictly.

After boiling for the specified time, the vessels are cooled to 40° C. While they are cooling, 2 ml. of 0·5N sodium hydroxide are introduced into the extraction chamber (see p. 536). The reaction vessel is then attached to the side-arm o of the manometric apparatus by a short length of heavy rubber tubing so that the vessel is in a nearly horizontal position with a large area of solution exposed.

The carbon dioxide is next transferred to and from the Van Slyke-Neill chamber as described on p. 547. Only ten transfers are required, but the vessel must be kept at about 40° C. and shaken by hand fairly vigorously to ensure complete extraction of carbon dioxide. The carbon dioxide is finally regenerated and measured exactly as described in the previous example (p. 551 *et seq.*).

A "C" correction should be determined by blank analysis for each batch of 0·5N sodium hydroxide reagent and at any time when it may be suspected that this reagent has been exposed to carbon dioxide. If it is of good quality, the ninhydrin need not be included when making the blank analysis.

Checking the Reagents. The factors given in Table IV (p. 583) are based on the original formulae of Van Slyke and Sendroy. Glycine gives theoretical results at pH 4·7 and 95% of theory at pH 2·5. Most amino-acids give theoretical results at pH 2·5, the important exceptions being glycine, tryptophane, and cystine (90–95%), and lysine which gives 105%. Alanine is the most convenient reference substance.

Example 5: Estimation of Amino Groups with Nitrous Acid.*

While not as specific as the ninhydrin procedure (p. 556), decomposition with nitrous acid is widely used for the estimation of "amino nitrogen" in amino-acids and in other primary *aliphatic* amines.

In the absence of ammonia or urea, quantities of material containing not less than 0·0005 mg. and not more than 0·50 mg. of amino nitrogen can be analysed satisfactorily in the standard Van Slyke apparatus.

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* Van Slyke, D. D., *J. Biol. Chem.*, 1929, **83**, 425.

The precision of analysis is about 1% for a 5 ml. sample containing as little as 0·01 mg. per millilitre of amino nitrogen.

PRINCIPLES. The amine solution is acidified and freed from gas in the extraction chamber of the Van Slyke manometric apparatus. Sodium nitrite is then added and the nitrous acid so produced reacts quantitatively according to the equation:

$$RNH_2 + HNO_2 = ROH + H_2O + N_2$$
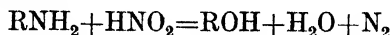
The nitrogen is extracted from the solution together with a much larger amount of nitric oxide produced by decomposition of the nitrous acid. This mixture of gases is transferred to a modified Hempel pipette and freed from nitric oxide by treatment with alkaline permanganate. The pure nitrogen is returned to the extraction chamber and its pressure is measured.

APPARATUS.

(1) *Van Slyke-Neill manometric apparatus.*

(2) *Modified Hempel pipette* (fig. VI.16).

(3) *Stop-cock pipettes* (fig. III.3, *a*, p. 145).

REAGENTS.

1. *Sodium nitrite, a cold saturated solution.*

2. *Glacial acetic acid.*

3. *Alkaline permanganate.* Shake 50 g. of crystalline potassium permanganate in 1 l. of 10% sodium hydroxide. Filter and store in a glass-stoppered bottle.

4. *Capryl alcohol or tri-butyl citrate.*

PROCEDURE. The aliquot of amine solution, which may be neutral or alkaline, should be such that a volume between 1 and 8 ml. contains a total of not less than 0·05 nor more than 0·5 mg. of amino-nitrogen. For optimum results a 5 ml. sample containing about 0·2 mg. of nitrogen is recommended.

(i) The extraction chamber *a* (fig. VI.12) is filled with mercury, one or two drops of "anti-foam" are placed in the capillary below cup *c* and 2 ml. of glacial acetic acid in cup *c*. A stop-cock pipette is filled with the solution, fitted with a rubber tip, and placed firmly in the bottom of the cup. The sample is then drawn into the chamber by the technique already described (p. 547), and is followed by 1 ml. of glacial acetic acid from the cup. After sealing the bore of the cock with mercury, the chamber is evacuated and shaken for 2 minutes at 300 to 400 oscillations per minute. The extracted gas is ejected, without loss of solution, as described on pp. 549–550.

(ii) The cup is then washed and 2 ml. of the nitrite solution are introduced into the chamber. The cock is sealed at once and the mercury is lowered until its meniscus is about 2 cm. above the bottom of the chamber. The solutions are allowed to react for the requisite time, which depends upon the actual volume and on the reaction temperature and may be determined

from fig. A.2 of p. 584. *Shaking is only carried out during the last minute of this period.*

Since a very large volume of gas is evolved, it is necessary to re-admit mercury from the reservoir from time to time to prevent the solution from being forced below the 50 ml. mark. During the shaking period the mercury should not be allowed to swirl in the bottom of the chamber.

(iii) While the reaction is taking place, a modified Hempel pipette is filled with alkaline permanganate solution up to the level of the three-way tap. The cup of the Hempel pipette and its outlet capillary, including the bore of the cock, are filled with distilled water and the cock is turned to an "off" position.

(iv) After the specified reaction time has elapsed, the mercury reservoir is raised and by manipulating the main control cock *j* (fig. VI.12) the gas is compressed under slight positive pressure in the upper part of the reaction chamber. The gas pipette containing the permanganate is fitted with a rubber tip and pressed firmly in the base of the cup above the chamber. With the reservoir at position *2*, the control cock is opened and the cock of the Hempel is turned to connect the Van Slyke chamber with the bulb of the pipette. By cautious opening of the two-way cock *b* the gas is forced into the gas pipette until the nitrite solution *just reaches* its cock. It is undesirable to let nitrite solution into the permanganate since this becomes exhausted too quickly. Both cocks are closed and the Hempel pipette is removed. The three-way cock of the gas pipette is now turned so that the gas remaining in the capillary between the cock and the bulb is driven into the latter by running in 1–2 ml. of water from the cup. After this, the cock is turned through a further 120° so that the nitrite is washed from the outlet capillary by more water from the pipette cup. The pipette is then shaken gently for about 30 seconds and put aside to complete the absorption of nitric oxide.

(v) The extraction chamber of the manometric apparatus is washed free from nitrite by introducing about 20 ml. of water, but no air. The water is ejected and the process is repeated with a further 10 ml. of water. Then 10 ml. of water are drawn into the chamber and freed from gas by the usual evacuation technique (p. 545). The mercury reservoir is returned to station *2* and the extracted air is expelled from the chamber together with 1 ml. of water which is allowed to remain in the cup. The rubber-tipped outlet of the gas pipette is again placed firmly in the cup of the Van Slyke apparatus and with cock *b* open the control cock is manipulated to drive a little water through the Hempel capillary and cock into its cup. The three-way cock is then rotated to put capillary and bulb in communication, and a *small* amount of water is likewise driven into the permanganate. The control cock of the apparatus is then closed and the reservoir lowered to station *3*. Next, by careful manipulation of the control cock, the

nitrogen gas is drawn from the Hempel into the extraction chamber. Care should be taken not to let more than a trace of permanganate enter the chamber. Both cocks are closed and the Hempel is removed.

(vi) A few millilitres of mercury are placed in the cup c (fig. VI.12), any trapped air bubbles are dislodged, and enough of the mercury is run through the cock to clear it of permanganate, after which it is closed.

The water meniscus is then lowered to the 2 ml. mark (or the 0·5 ml. mark if appreciably less than 100 mm. pressure is registered at 2 ml.) and the reading is taken.

Finally the gas is ejected without loss of solution (pp. 549–550), the meniscus again lowered to the mark, and the new reading of the vapour pressure of the water is noted (P_0). Then

$$P_{[N_2]} = P_1 - P_0 - C$$

The correction "C" compensates for errors due to nitrogen dissolved in the reagents and is found by conducting a blank analysis in which water replaces the test solution.

It is desirable to run a blank analysis before each series of determinations since this serves both to check the correction factor and to ensure that the apparatus is working properly.

By reference to Table V (p. 585), the concentration of amino nitrogen in the sample can be obtained from the measured value of $P_{[N_2]}$.

TABLE OF

FURTHER GASOMETRIC METHODS USING THE VAN SLYKE APPARATUS

Estimation	Reference
1. Bicarbonate content of blood and urine.	"Quantitative Clinical Chemistry," Peters and Van Slyke (Bailliere, Tindall and Cox, London, 1932), p. 292.
2. Plasma carbon dioxide combining capacity.	Van Slyke, D. D., and Cullen, G. E., J. Biol. Chem., 1917, **30**, 289.
3. Methaemoglobin in blood.	Van Slyke, D. D., and Hiller, A., J. Biol. Chem., 1928, **78**, 807.
4. Urea by urease.	Van Slyke, D. D., J. Biol. Chem., 1927, **73**, 695.
5. Iodates, sulphates, and total base in blood serum.	Van Slyke, D. D., Hiller, A., and Berthelsen, K., J. Biol. Chem., 1927, **74**, 659.
6. Reducing sugars in blood.	Van Slyke, D. D., and Hawkins, J., J. Biol. Chem., 1928, **79**, 739.
7. Calcium in blood.	Van Slyke, D. D., and Sendroy, J., J. Biol. Chem., 1928, **84**, 217.
8. Lactic acid in blood.	Avery, B., and Hastings, A., J. Biol. Chem., 1931, **94**, 273.
9. Determination of potassium.	Kramer, B., and Gittleman, I., Proc. Soc. Exper. Biol. and Med., 1926, **24**, 241.

B. ANALYTICAL USES OF THE WARBURG APPARATUS

The name "Warburg apparatus" is commonly applied to a type of respirometer devised originally by Barcroft and Haldane* in 1902 and then termed a "blood-gas manometer." Since 1923 it has been used extensively by O. Warburg† and his school at Berlin for studies of tissue respiration, and in recent years has become a general analytical tool in biochemical laboratories. Like the Van Slyke manometric apparatus, the Warburg manometer is a versatile instrument which, if more widely used, would find innumerable applications in other fields.

The usual form of Warburg manometer and reaction vessel is illustrated in fig. VI.23. It operates on the "constant volume" principle, so that any changes of gas concentration occurring in the closed system resulting from reactions taking place in the flask may be observed and measured as pressure changes in the manometer.

Construction of Apparatus

(1) *Manometer*. The **U**-tube (fig. VI.2 3, *A*) is made from precision bore tubing of 1 sq. mm. cross-sectional area as nearly as possible, and of about 8 mm. outside diameter. Both limbs carry accurately aligned graduations at 1 mm. intervals for a length of 30 cm., with the zero mark usually at the lower end, but sometimes in the centre. A side-tube is fused to the bend and carries a short length of pressure tubing, closed at the end and capable of being compressed by the stout screw-clamp *C*, which is fitted with a small plate to increase the area of rubber compressed.

The left-hand limb of the manometer is open and the right-hand limb is closed by a **T**-cock *T* as used in vacuum technique. About 3 cm. below the cock, a side-arm *S* extends horizontally and at 90° to the plane of the **U**-tube for some 10 cm., where it is bent parallel with the manometer for a further 6–10 cm. and terminates in a ground cone-joint *J*.
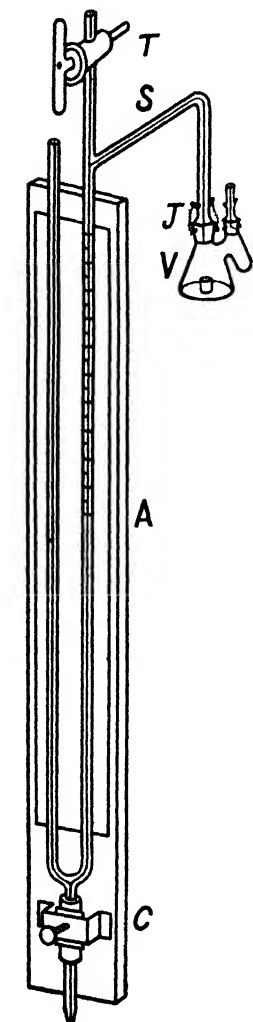
Fig. VI.23.

Warburg's Manometric Apparatus. General Design of Manometer and Flask.

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* Barcroft, J., and Haldane, J. S., *J. Physiol.*, 1902, **28**, 232.
† Warburg, O., *Biochem. Z.*, 1923, **142**, 317.

(2) *Reaction Vessel.* The reaction vessel (fig. VI.24) may be of any convenient size, but is commonly of about 15 ml. capacity. A small circular cup *d* is fused to the bottom of the vessel and is about 12 mm. deep by 10 mm. diameter. The side vessel *e* has a capacity of about 1 ml. and is accessible from the vent *f*. This is fitted with a combined cock and stopper *g** so arranged that its capillary is put in communication with the vessel by rotation through 90°.

(3) *Support.* The manometer and reaction flask are fixed to a rigid support capable of being attached to the shaking mechanism (see below). The support may be of wood, monel metal, or stainless steel, and should be fitted with a mirror to assist in avoiding parallax.

(4) *Water-bath and Thermostat.* During a determination, the reaction vessel must be *completely* immersed and shaken in a water thermostat. In order to obtain consistent results it is essential that the thermo-regulator be sufficiently sensitive to keep the temperature constant within 0·1° C. or better. Since effective temperature control depends upon thorough mixing of the water, a stirrer of adequate power is required. One motor may serve both the stirrer and the shaker.

Fig. VI.24. Reaction Vessel.

(5) *Shaking Mechanism.* Some types of shaker are designed to move the flask through an arc of 4–6 cm. and others to impart a parallel motion of about the same amplitude. There is little to choose between the two, but the parallel type is more easily constructed to maintain all the manometers in vertical alignment.

(6) *Number of Units.* The apparatus may be designed to take any number of manometer-flask units. A minimum of two is required, since one is needed as a thermo-barometer. Except in special circumstances, twelve units (six each side of water-bath) represents an upper limit both as regards expense and convenience.

Operational Technique

1. *Assembling the Apparatus.* The ground joints must be gas-tight, spring fitted, and greased as described on p. 539. Scrupulous cleanliness must be observed; in particular the U-tube must be quite grease-free since it is of narrow bore and is usually filled with an aqueous solution.

Since a constant is determined for each reaction vessel/manometer assembly (see below), care should be taken to number each pair and to see

* Warburg, D., and Kubowicz, F., *Biochem. Z.*, 1929, **214**, 5.

that they are kept together. If a flask or manometer should be damaged and replaced, a new constant must be determined.

2. *Filling the Manometer.* The fluid generally used is "Brodie's solution," prepared as follows:

Sodium chloride, 23 g.: Sodium tauroglycocholate, 5 g.: Thymol, 0·5 ml. of saturated alcoholic solution: Dye (e.g. methyl violet or Evans' blue), 0·1 g.: Water to 500 ml.

10,000 mm. of Brodie's solution exerts a pressure approximately equal to 760 mm. of mercury. Other liquids, e.g. dibutyl phthalate, can of course be used, and are sometimes advantageous.

The solution is best introduced through the rubber reservoir at the bend of the **U**-tube, and the quantity should be such that with the rubber half compressed, the level in the two limbs is about 150 mm.

3. *Reading Manometers.* The readings are significant to $\frac{1}{2}$ mm. if parallax is avoided and the manometers are truly vertical. The mirror glass scale is of great assistance in avoiding parallax, but where such scales are fitted, a reference mark must be etched on the manometer tube so that its proper relation to the scale can be maintained. Three such marks, at 0, 150, and 300 mm., are better still and simplify determination of "vessel" volumes.

If the shaker is the type where the flask moves through an *arc*, care must be taken to centralise the manometers before making a reading.

When making the actual reading, it is essential to make sure that the meniscus is quite free. The most satisfactory method is to *raise* the meniscus about 1 cm. above the reference mark and then to lower it again to the mark, at which point a sharp tap on the rubber reservoir will ensure that the meniscus is free and any slight readjustment can then be made.

4. *Filling Vessel with Gas.* In most determinations the atmosphere in the flask has to be replaced by a gas other than air. This gas is led in *via* the cock at the top of the manometer and flows through the vessel, finally to emerge from the Warburg-Kubowicz stopper. Cylinders of compressed gas are generally utilised, and in such cases it is necessary to include both an efficient reduction valve and a safety valve in the circuit to prevent the gas pressure from forcing the Brodie solution out of the manometer.

A simple safety valve is shown in fig. VI.25. The fluid may be either mercury or water, the latter being preferred since the required pressure is usually less than 10 cm. of water.

To avoid evaporation of solvent from the flask, the gas must be saturated by passing it through a suitable bubbler containing the same solvent (usually water) and maintained at thermostat temperature.

While the gas is flowing through the system it is necessary to squeeze the manometer reservoir from time to time to displace the column of air remaining above the fluid in the right-hand limb. The squeezing should be sufficient to force the Brodie solution almost to the level of the **T**-junction.

Since the air must be replaced by the other gas while the flask is fully immersed in the thermostat, it is convenient to fit a 2 in. length of rubber tube to the stopper outlet so that the gas emerges well above the surface of the water. In order to prevent back-diffusion of air when the gas flow is stopped, the rubber tube should terminate in a 1 in. length of glass capillary. If hydrogen or other light gas is in use, the capillary should be bent to a semicircle *so that the orifice is downwards.*

5. *Use of Centre Cup.* It is characteristic of the Warburg manometer that changes in the concentration of *one* gas only may be measured in any one experiment. Thus, in tissue respiration experiments oxygen may be taken up and carbon dioxide evolved at the same time; to follow the oxygen uptake it is necessary to remove the carbon dioxide, and the primary function of the centre cup is to hold absorbents for such interfering gases. In the case quoted carbon dioxide can be absorbed by fitting a roll of starch-free filter-paper into the centre cup so that 5–7 mm. of paper project from it. The paper is saturated with 20% caustic potash solution.

The centre cup can also be used as the reaction vessel (see example 2), but where only one gas is absorbed or evolved it is left empty.

6. *Shaking Speed.* In most cases a speed of about 100 oscillations per minute is to be recommended, but this may of course be varied to suit a particular experiment.

Fig. VI.25.
Safety Valve for Use with Gas Supply.

It must be emphasised, however, that where a rate of reaction is being followed, the speed must be kept constant so that the conditions are alike in comparative experiments, though as a matter of principle every effort should be made to make the experimental conditions independent of the shaking speed.

7. *Lubrication of the Ground Joint between Manometer and Flask.* This joint needs special treatment, for if the joint is greased in the ordinary way and then placed in a warm water-bath, the pressure of the retaining springs (fig. VI.23, *J*) is sufficient to cause the lubricant to flow and the flask is then forced further on to the manometer cone, so leading to an apparent increase in gas pressure. To avoid this error, Dixon* recommends anhydrous lanoline as the lubricant. After applying three or four spots to the cone,

* Dixon, M., "Manometric Methods," 2nd ed. (Cambridge Univ. Press, 1943).

the flask is pressed on gently until a homogeneous joint is obtained. After immersion in the warm thermostat for a few minutes, the flasks are rotated a few degrees in each direction until the joint *just* binds; no further movement then occurs.

In some cases (see p. 571) special lubricants may be required.

CALCULATIONS.

1. *Principles.* Before making a reading, the volume of the gas system, i.e. the vessel, connecting tube, and upper part of the manometer, is adjusted to a constant figure by manipulating the screw clamp. For most purposes the 150 mm. mark (or 0 mm. in the case of a centre-zero scale) on the right-hand limb is used as a datum.

The level in the left-hand (open) limb then indicates the difference in pressure between the atmosphere and the vessel contents.

It is conventional to express all volumes in *cubic millimetres* and pressures in millimetres of the manometer fluid. Gas volumes are measured in terms of *dry gas* at N.T.P.

For any apparatus these relations are expressed in the formula:

$$x = h \left[\frac{V_g \dfrac{273}{T} + V_f(\alpha)}{P_0} \right] \quad \dots\dots\dots\dots\dots(1)$$

where

$x =$ (cubic millimetres) of gas at N.T.P.

NOTE.—x is *negative* if gas is absorbed; *positive* if gas is evolved.

$h =$ change of manometer reading in millimetres.

$V_g =$ free volume of the gas space.

$T =$ absolute temperature of the thermostat.

$V_f =$ volume of liquid in the vessel.

$\alpha =$ solubility of the gas being measured in the liquid present, at $T°$ and at a partial pressure P_0.

$P_0 =$ normal pressure in millimetres of *manometer fluid*. If the fluid has a density D then

$P_0 = 760 \times \dfrac{13 \cdot 60}{D}$.

Consideration of the equation (1) shows that for a given apparatus, used at the same temperature and with the same total volume of liquid, the expression in square brackets remains constant.

This is known as the "apparatus constant," k. Thus

$$x = h \cdot k \text{ for a given gas} \quad \dots\dots\dots\dots\dots\dots(2)$$

In the above calculation it has been assumed that only one gas was present. If, however, other gases are present *and do not enter the reaction,*

their amounts, partial pressures, and fractions in solution remain constant and cancel out, leaving the equation still valid.

2. *Determination of the Constant, k.* The commonest and most accurate method is that of substitution of the appropriate values in the formula.

Thus T and V_f are known, while α can be obtained from published tables. P_0 requires an accurate determination of the density of the manometric fluid, and V_g is obtained by subtraction of V_f from the total volume of the reaction vessel, etc.

The total volume of the vessel and the part of the manometer to be filled with gas is found in the following way: A mark is made 2-3 cm. above the cone-joint J (fig. VI.23) and the dry manometer is inverted. Clean, dry mercury is then introduced until the space between the datum line and the mark on the stem is full and free from air bubbles. It is necessary to tilt the manometer during this operation so that the two marks are in horizontal alignment. The mercury is then removed and weighed.

Next, the dry reaction vessel is filled with mercury to such a level that, on fitting the cone-joint, which should be lubricated, the mercury is forced up exactly to the mark on the stem. From the combined weight of mercury the volume may be calculated. In doing this the temperature correction should not be forgotten.

Other methods sometimes used are:

1. The Münzer and Neumann* method, in which measured amounts of gas are introduced to the system.

2. Quantitative liberation of gas in the apparatus, typical reactions being:

(*a*) for carbon dioxide—mixing of known amounts of acid and bicarbonate.

(*b*) for oxygen—mixing of hydrogen peroxide and potassium permanganate;

(*c*) for nitrogen†—mixing of excess alkaline hydrazine with potassium bi-iodate.

It should be emphasised that the constant should be determined separately for each reacting gas† and for all values of V_f. McCleod and Summerson‡ have published a graphic method for determining constants at different values of V_f, which largely eliminates a tedious series of calculations.

3. *The Thermo-barometric Correction.* Since the Warburg apparatus is, in effect, a sensitive air thermometer, it follows that very slight changes in temperature will affect the readings. Accordingly, with every series of analyses it is necessary to include a spare manometer and flask. The vessel

* Münzer, E., and Neumann, W., *Biochem. Z.*, 1917, **81**, 319.

† Actually the solubilities of N_2, O_2, and CO are so nearly alike that the constants are interchangeable. Method 2 (*c*) is capable of quite accurate results and is very simple.

‡ Summerson, W. H., *J. Biol. Chem.*, 1939, **131**, 579; MacLeod, J., and Summerson, W. H., *Science*, 1940, **91**, 201.

need not contain the reagents used in analysis, but must contain the same solvent.

Any changes of temperature or pressure during an analysis are registered on the "thermo-barometer," and since the other manometers are affected equally, it is only necessary to subtract this reading from those on the test vessels.   When making this subtraction the sign of the change must be borne in mind.

REPRESENTATIVE ANALYTICAL PROCEDURES

Example 1: Micro-hydrogenation (e.g. Determination of "Double Bonds").

PRINCIPLES. The Warburg manometer is eminently suitable for following hydrogenation reactions and several quantitative methods have been described, most of which make use of solid or suspended catalysts and sometimes involve operation at elevated temperatures.   In addition to water, ethyl alcohol, acetic acid, *cyclo*-hexane, chloroform, *cyclo*-hexanol, and, less conveniently, octyl alcohol and decalin can be used as solvents, but these all need most careful purification before use, and it is always essential to carry out simultaneously a blank experiment with a vessel containing solvent only. Solvents of particularly high vapour pressure, such as chloroform or methyl alcohol, should be avoided if possible, since condensation in the manometric liquid may occur and exceedingly good temperature control would be needed to prevent appreciable variations of the internal pressure.   The manometric liquid should be selected with care.   Thus water or Brodie's solution should not be used with hydrocarbon solvents; decalin and dibutyl phthalate are useful choices amongst liquids of very low vapour pressure.

Amongst the many catalysts that can be used, colloidal platinum and colloidal palladium are generally the most suitable.   Details are given below for operation with a colloidal palladium catalyst, which has been found to be far superior to any other for the hydrogenation at room temperature of water-soluble substances such as fumaric or crotonic acids.[*]

A milligram of an unsaturated substance of molecular weight *ca.* 100 and containing one double bond requires about 200 cu. mm. of hydrogen for complete reduction, and this is within the range of a standard Warburg manometer having a vessel constant between 1 and 3 (see p. 566).   The error in dealing with amounts of material between 0·1 and 1 mg. is less than 2%.

In order to compensate empirically for incomplete reduction of substances such as poly-enes, in which it is important to discover the total number of double bonds present, Kuhn and Möller[†] have proposed that a differential

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[*] Harrison, K., *Biochem. J.*, 1939, **33**, 1465.
[†] Kuhn, K., and Möller, E. F., *J. angew. Chem.*, 1934, **47**, 145.

method should be used, comparison being made with a known pure substance of similar chemical character (sorbic acid has been suggested). In this case, however, a form of Barcroft's differential manometric apparatus (pp. 574–578) is more convenient. The use of empirical methods such as the above is to be deprecated in quantitative micro-analysis, and should be avoided whenever possible.

Method for the Hydrogenation of Water-soluble Compounds

APPARATUS. Warburg manometric apparatus fitted with hollow-stoppered vessels (p. 563).

REAGENTS.

1. *Colloidal palladium catalyst.** Dissolve 0·2 g. of palladium chloride in 100 ml. of warm water, add 0·2 g. of gum arabic, cool, and filter into a brown bottle. The salt is reduced to the metal during the equilibration period preceding each determination (see below).

2. *Hydrogen gas.* Cylinder hydrogen is satisfactory, but the usual reduction valve should be supplemented by a mercury safety valve (see p. 565).

3. *N hydrochloric acid.*

Calibration of the Manometer. Since gas is *absorbed* the volume of the "vessel" should be measured from a point high on the closed limb. This enables almost the full excursion of the manometer to be used.

PROCEDURE. The substance to be examined is dissolved (in water) to make an approximately $M/200$ solution. 2 ml. of this solution are placed in the main vessel, followed by 1 ml. of N hydrochloric acid.† 0·5 ml. of the palladium chloride solution is then placed in the side cup of the vessel and the hollow stopper is fitted in position. This stopper should be fitted with the anti-diffusion device described on p. 565.

One or two more vessels should be prepared similarly for replicate determinations and a further vessel must be filled with a few millilitres of water to serve as a thermo-barometer.

The vessels are next attached to their respective manometers, connected with the shaker, and placed in the water-bath. Hydrogen is now passed through all the vessels at about 100 ml. per minute, while the manometers are shaken at 200 oscillations per minute. The gas is allowed to flow for 15–20 minutes, and during this period it is necessary to replace the air in the capillary between the vessel and manometer fluid with hydrogen, as already described (p. 564).

The main cock T (fig. VI.23) and the hollow stopper of each manometer are now closed, the hydrogen supply is disconnected, and shaking is carried out

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* Skita, A., and Franck, H. H., *Ber.*, 1911, **44**, 2862.

† The acid accelerates hydrogenation considerably, but neutral solutions may be used when necessary.

for a further 10 minutes to make certain that the catalyst is fully saturated and to equilibrate the system. Equilibrium is attained when the test manometers and the thermo-barometer all show no significant movement within two or three minutes. At this stage each manometer-vessel assembly is removed from the bath and the catalyst is tipped from the side-arm into the main part of the vessel. The apparatus is then replaced in the bath and the fluid in the right-hand limb of each manometer adjusted exactly to zero* by means of the reservoir screw. After this adjustment, the reading of the left-hand (open) limb of each manometer, including the thermo-barometer, is taken and recorded (h_1; h_{t_b}). Shaking is then recommenced, and if the compound is fairly reactive, reduction proceeds to completion in 20–30 minutes.

During this period of hydrogen uptake it is necessary to stop the shaker from time to time and re-adjust the level of fluid in the right-hand limb approximately to zero.

When reduction is complete, as indicated by a steady manometer reading, the shaker is again stopped, the right-hand meniscus adjusted *exactly* to zero and the new reading of the left-hand limb of the manometer is recorded (h_2). Then,

$$(h_1 - h_2 - h_{t_b}).k = \text{cubic millimetres of hydrogen taken up}$$
$$\text{by the sample at N.T.P.}$$

The constant k is, of course, determined for each manometer by substitution of the appropriate values in the equation given on p. 566.

Example 2: The Autoxidation of Fatty Oils.

The procedure of Johnston and Frey† for studying the accelerated autoxidation of fats and oils illustrates the use of the Warburg apparatus in an industrial sphere. It also demonstrates the practicability of using Warburg manometers at elevated temperatures.

PRINCIPLES. An unsaturated oil is placed in the central cup of a special vessel (fig. VI.26) so that a 1 mm. layer is formed. The system is filled with oxygen and the oil is shaken in this atmosphere at 100° C. The rate of oxygen uptake is followed until 1,500 cu. mm. of oxygen have been absorbed and the results are graphed.

For most autoxidisable substances there can be observed the occurrence of an "induction period" which precedes any rapid absorption of oxygen. For commercial oils (e.g. sesame oil, corn oil, or cottonseed oil) free from any added catalyst, Johnston and Frey† define this period as the time taken for 0·5 ml. of oil to absorb 1,000 cu. mm. of oxygen at 100° C. and consider this figure to be reproducibly diagnostic of the quality of a commercial fatty oil. Results consistent to within 1% are claimed.

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* i.e. the mark from which the vessel volume has been determined.
† Johnston, W. R., and Frey, C. H., *Ind. Eng. Chem.* (Anal. Edn.), 1941, **13**, 479.

APPARATUS.

1. *Warburg Manometers with Special Flasks* (fig. VI.26). The flasks have about 70 ml. capacity and are blown with centre cups 24 mm. in diameter and 20 mm. high. Since the surface area exposed to the oxygen should be constant, a series of standardised flasks are required. Warburg-Kubowicz stoppers with capillary outlets are needed, but not side vessels.

The flasks should be washed in carbon tetrachloride and dried carefully before use.

2. *Thermostat controlled at* $100°$ C.± 0.05. 20% ethylene glycol in water is a convenient liquid to use.

REAGENT.

A cylinder of oxygen, fitted with reduction and safety valves (p. 565).
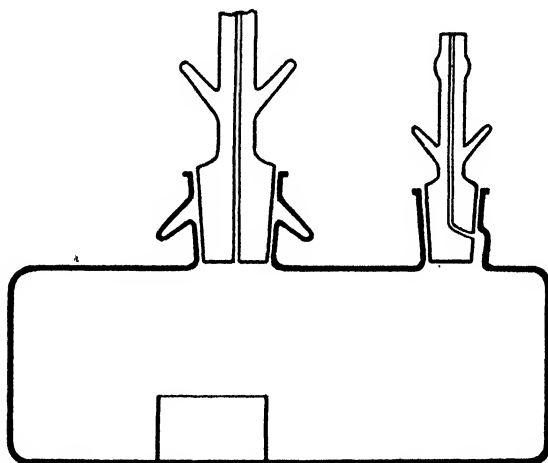
Fig. VI.26. Reaction Vessel for Autoxidation of Fatty Oils.

PROCEDURE. As in Example 1 (pp. 568–570) the "vessel volume" for calculation of the constant (K_{O_2}) should be determined from a zero mark at the top of the closed limb of the manometer.

0.5 ml. of the oil to be examined is pipetted into the central cup and the flask is attached to the manometer, the joint being lightly lubricated with *high-vacuum grease* free from unsaturated compounds.

While the flask and contents are still at room temperature, oxygen is passed through the vessel, at a pressure of between 10 and 15 cm. of Brodie solution, for about 15 minutes. Then the cock above the manometer is closed, but the outlet is left open and the flask is transferred to the thermostat to equilibrate without shaking for a further 10 minutes. During equilibration the manometer fluid is raised to a little less than the zero mark and is

adjusted exactly to it just before closing the outlet, at the end of the equili-
bration period.

A thermo-barometer, the flask of which is quite dry, is placed in the bath
together with the test vessels.

Shaking is then commenced at a standardised rate of 110–120 oscillations
per minute and an amplitude of 3 cm. This reduced amplitude avoids
splashing and maintains a 1 mm. layer of oil over the bottom of the cup.

Pressure readings are taken at intervals of from 5–30 minutes, depending
on the particular oil under test. The determination is normally concluded
when the Brodie solution has reached the bottom of the left-hand limb,
but in this instance the manometers are reset by allowing a little extra
oxygen into the system and further observations are then carried out.

Example 3: Determination of Choline-esterase Activity of Serum.*

By following the output of carbon dioxide, the Warburg apparatus can
be used for studying any reaction (within the range of pH given by bicarbonate
buffer systems) in which hydrogen ions are formed.

The method given below illustrates the study of an enzyme reaction
involving acid production in a bicarbonate buffer solution.

PRINCIPLES. The enzyme *choline-esterase* hydrolyses acetyl choline to
choline and acetic acid. The reaction is maximal at pH 8·4 and only
slightly slower at pH 7·0. This is within the range of an $H_2CO_3/NaHCO_3$
buffer and the reaction is usually carried out in $0·0225M$ sodium bicarbonate
in equilibrium with a gas mixture of 5% CO_2/95% N_2, since the bicarbonate
concentration is then equal to that of normal plasma bicarbonate whilst
the pH is that of blood (7·4).

Comparison of the enzymatic activity of different sera can thus be made
by mixing them with acetyl choline in the bicarbonate buffer and observing
the carbon dioxide output over a standard time (usually 30 or 60 minutes
from the time of mixing).

The *order of reaction* can be deduced from the nature of the gas evolution/
time curve and the *velocity of reaction* from the slope of the curve.

The chosen example gives a straight line curve, indicating a "zero order"
reaction.

REAGENTS.

1. $0·03M$ *sodium bicarbonate in water.*

2. $0·1M$ *acetyl choline chloride*† *(or bromide) in water.*

3. *Gas mixture of* 95% *nitrogen and* 5% *carbon dioxide contained in a
steel cylinder.*

---

* Ammon, R., *Archiv. ges. Physiol.* (*Pflüger's*), 1933, **233**, 486.
† Acetyl choline readily hydrolyses in aqueous solution especially if alkaline. How-
ever, if the solution is prepared in water saturated with carbon dioxide and stored in
the refrigerator, it keeps well for 2 or 3 days.

Calibration of the Manometer

Since gas is *evolved* in this reaction, it is convenient to determine the "vessel volume" from a point *low* on the closed limb of the manometer. This allows the operator to follow active preparations for a longer time, since pressures up to 250 mm. of Brodie solution can be recorded. The constant should be determined for a fluid volume (V_f) of 3·5 ml.

PROCEDURE. 2·5 ml. of 0·03M sodium bicarbonate are pipetted into the main part of the Warburg flask, followed by 0·5 ml. of 0·1M acetyl choline solution.

0·5 ml. of serum is placed in the side-arm of the flask and the latter is attached to the manometer in the usual way.

In addition to a thermo-barometer, a third Warburg flask is prepared in which the same reagents are placed in the main well, but water in place of serum in the side-arm. This serves as a blank to compensate for a small amount of spontaneous hydrolysis of the acetyl choline which occurs during the experiment.

When all the test flasks, the thermo-barometer, and the "blank" are prepared, they are placed in the thermostat at 38° C. and filled with the nitrogen/carbon dioxide gas mixture as described on p. 564. During the passage of the gas the flasks should be shaken at the usual rate. To facilitate this process, the apparatus should be fitted with a multi-way gas manifold, so that all flasks can be gassed simultaneously.

After 10 minutes the gas supply is disconnected, the cocks are closed, and a further 5 minutes' equilibrium period is allowed. At the end of this time the readings are taken and the serum is tipped into the acetyl choline/ bicarbonate mixture. Further readings are made at 10 minute intervals for 1 hour, and after subtracting the thermo-barometer differences the carbon dioxide output can be obtained for each test by multiplying h by $K_{[CO_2]}$.*

It only remains to subtract the appropriate amounts of gas produced in the "blank" and to plot the results as carbon dioxide output against time. A straight line should be obtained. The volume of carbon dioxide produced in 1 hour is a measure of the activity of the sample. Each test should be made in duplicate at least.

* For calculation see p. 566.

ANALYTICAL USES OF THE BARCROFT DIFFERENTIAL RESPIROMETER

The essential parts of this apparatus are shown in fig. VI.27. The illustration is of a modern form recommended by Dixon and Elliot,* and differs from the original design of Barcroft† in the disposition of the taps.

While similar to the Warburg apparatus, the Barcroft respirometer has two similar flasks, one attached to each limb of the U-tube. No provision is made for varying the volume of the manometric fluid. In some circumstances the apparatus has advantages over the Warburg manometer and deserves consideration by those workers interested in the kinetics of reactions in which a gas is evolved or absorbed.
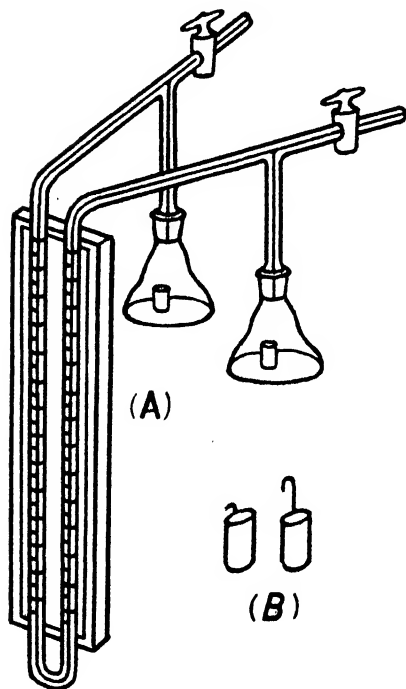
Fig. VI.27.
The Barcroft Differential Respirometer.
A, General diagram. B, Small vessels for extra reagents.

Construction

1. *Manometer*. The U-tube is made from capillary tubing of about 7 cm. outside diameter. The bore of the capillary is generally less than 1 sq. mm. in area and should be as uniform as possible. The parallel limbs of the U-tube are about 25 cm. long, and may be graduated at 2 mm. intervals or left plain for use with a separate scale. The top of each limb is bent at right-angles‡ to the plane of the U-tube and terminates in a capillary-bore tap. About 10 cm. from the bend a T-tube is fused in each limb to carry the cone-joint for the reaction vessels, and is of such a length that the flasks can be immersed completely in the water-bath.

2. *Vessels*. The two vessels for each manometer are usually of 30–40 ml. capacity, and should be equal in volume to within 0·1 ml. Each is fitted

---

* Dixon, M., and Elliot, K. A. C., *Biochem. J.*, 1930, **24**, 820.
† Barcroft, J., *J. Physiol.*, 1908, **37**, 12.
‡ The older patterns of Barcroft apparatus have the plane of the U-tube at an angle of about 110° with the top horizontal parts. This is undesirable, and complicates the calculations (see p. 576).

with a central cup, similar to that used in the Warburg apparatus. A reduction in the size of the vessels increases the sensitivity, and flasks as small as 5 ml. capacity can be used.

For the addition of reagents during an experiment, small tubes (fig. VI.27) made of soft glass tubing with platinum wire hooks fused into the edge can be used. Fig. VI.28 illustrates a special flask due to Dixon and Keilin* which is fitted with a side vessel and stopper of the Warburg-Kubowicz type (compare fig. VI.24, p. 563) and a hollow cock through which acid or alkali may be introduced.

3. *Assembly.* The remainder of the assembly is similar to that required for the Warburg apparatus. When using a number of units, especially if these are fitted with standard joints, it is particularly important to see that each set is numbered very clearly, otherwise confusion may easily arise and ruin many hours of work.

The manometric fluid in common use is paraffin oil coloured by a suitable dyestuff, such as Sudan III or Waxoline blue.

GENERAL TECHNIQUE. Most of the remarks on the Warburg technique (pp. 563–568) apply with equal force to the Barcroft apparatus.

When a particular gas mixture has to be introduced into the flasks, the most convenient procedure is to connect the open ends of the manometer together with a glass T-tube. The third limb of the T-tube is then connected to one arm of a three-way tap. The gas supply is led to one of the remaining arms of this tap and a vacuum pump is connected to the other one. Thus both flasks may be evacuated slightly, water-pump vacuum being sufficient, and since both flasks are equally affected no movement

Fig. VI.28. Dixon-Keilin Flask.

of the manometer fluid takes place. After evacuation the tap is turned so that the required gas enters the flasks. The process should be repeated three or four times to fill the flasks completely with the gas to be used.

CALCULATION. In the Barcroft apparatus the evolution or absorption of a gas in one flask causes both volume and pressure changes in the system. This fact complicates the theoretical calculation, but for most purposes the following simplified formula may be used.†

---

* Cf. Dixon, M., "Manometric Methods" (Cambridge University Press, 1943).
† For a fuller discussion reference should be made to Dixon's book on "Manometric Methods," in which a complete theoretical treatment is given.

$$X = h \left(\mathrm{Cos}\ \theta + \frac{A.P_0}{2V_{g'}} \right) . \left(\frac{V_g . \dfrac{273}{T} + V_f}{P_0} + \frac{A}{2} . \frac{273}{T} \right)$$

$$= h.k.$$

where X=volume of gas absorbed or evolved in cubic millimetres.

> h=difference of level of manometer fluid in millimetres.
>
> θ=the angle which the plane of the U-tube makes to the vertical in the reading position (0° in the design of fig. VI.28). This should be measured accurately.
>
> A=cross-section of the U-tube capillary in square millimetres. This should be less than 0·5 for the formula to be valid.
>
> P_0=normal pressure, in terms of manometric fluid.
>
> $V_{g'}$=total volume, in cubic millimetres, of the gas space in the compensating vessel, including the space in the manometer as far as the central zero mark.
>
> V_g=free volume of reaction vessel.
>
> V_f=volume of all liquids in which the gas might dissolve
>
> =solubility coefficient of the gas being measured in the liquid used (at temperature T).
>
> T=*absolute* temperature of the water-bath.

Determination of the Apparatus Constant (k). With soluble gases such as carbon dioxide, the only suitable procedure is that of substitution of the respective values for the relevant terms in the above formula. Vessel volumes should be measured by the method given on p. 567.

If oxygen or nitrogen are to be measured, then the method of Münzer-Neumann (ref. p. 567) can be used.

REPRESENTATIVE ANALYTICAL PROCEDURES

The Determination of Oxygen Absorption of Sewage and Sewage Effluents*

The following examples illustrate the potentialities of the Barcroft apparatus in technical analysis.

The "biochemical oxygen demand" of sewage is defined as the volume of oxygen taken up in 5 days by 100 ml. of the fluid. As usually conducted, this is a lengthy operation, requiring a good deal of space. With the method given below a good indication of the condition of sewage can be obtained in less than 24 hours. Furthermore, the rate of oxygen absorption can be followed continuously using quite small amounts of material.

---

* Wooldridge, W. R., and Standfast, A. F. B., *Biochem. J.*, 1936, **30**, 141–162.

An extensive comparison of the manometric and volumetric methods has been made by the originators of this procedure, and while it is not possible to derive any simple relationship between the figures obtained by the two methods, the manometric method gives sufficient information for assessing the condition of sewage in as little as 6 hours.

The procedure is again more flexible, since by virtue of the differential feature of the Barcroft manometer it is easy to follow the effect of changing one or more conditions in the sewage treatment without the necessity of conducting a large number of full-scale tests.

It has been suggested that a good effluent might be defined as one which does not give an absorption greater than 1 part in 10^5 after 24 hours at 22° C. in the Barcroft apparatus.

Example 1 : Direct Examination of Sewage.

PRINCIPLES. The sample of sewage is shaken with an excess of air at room temperature. The carbon dioxide produced is removed continuously by a solution of caustic potash contained in the central cup, and the oxygen uptake is followed on the manometer.

METHOD.

(i) *Determination of the Constant k.* The constant is determined for oxygen and for a fluid volume, V_f, of 3·3 ml. in each flask. Since it is usually difficult to maintain a thermostat at temperatures in the region of 20° C. it is convenient to calculate a series of constants for 1° intervals between 15° and 25° and to operate at room temperature. If a standard temperature is considered essential, however, then a thermostat at 25° should be used.

(ii) *Measurement of the Oxygen Absorption.* 3·3 ml. of water are placed in the left-hand flask of each manometer unit and 0·3 ml. of 10% caustic potash is pipetted into the central cup of each right-hand flask. 1·0 ml. of the sewage and 2·0 ml. of water are placed in the main part of each right-hand flask, though any suitable aliquot may be used, provided that the total volume in each vessel is equal to the V_f (3·3 ml. in the present example) for which the constant has been calculated.

The flasks are attached to the manometers and the units are placed in the water-bath with all taps open to give free access to the air.

Next the units are shaken at about 150 oscillations per minute for 15–20 minutes to ensure proper temperature equilibration. At this stage the shaker is stopped, the taps are closed, and the readings of each side of the manometer are at once noted. The shaking is then recommenced and readings are taken at suitable intervals, e.g. hourly.

The total oxygen absorption at each time interval is simply obtained by multiplying the observed reading (millimetres) by the constant k. The oxygen uptake, calculated in cubic millimetres per millilitre of sewage

multiplied by 0·143, gives the oxygen uptake as "parts of oxygen absorbed by 100,000 parts of sewage."

The results are graphed, and the curves so obtained are sufficiently distinctive as to allow an analyst to form a good opinion as to the condition of any sample of sewage.

Example 2: The Oxidation of Sewage in the Presence of Activated Sludge.

PRINCIPLES. Sterile sewage is placed in each of the Barcroft flasks after dilution with a suitable buffer. The system is equilibrated in the presence of air and then a washed suspension of the sludge is added to the contents of one flask. The oxygen uptake is followed as in the previous example.

REAGENTS.

1. *Potassium hydroxide, a* 10% *solution in water.*

2. *Phosphate buffer, p*H=7·4.

3. *Sterile sewage.* This is prepared by filtering a "strong" crude sewage through a Seitz bacterial filter. The sterile filtrate is then used.

METHOD. 0·3 ml. of the caustic potash is pipetted into the central cup of each flask, and an amount of sludge up to 2·5 ml. into *each* main vessel. If an amount of sludge less than 2·5 ml. is found necessary the difference is made up with distilled water.

0·5 ml. of the filtered sewage is put in a loose cup and this is attached to the right-hand central cup. 0·5 ml. of water is put in a similar loose reagent vessel and hooked to the cup of the left-hand flask. The flasks are then attached to the manometers and placed in the water-bath, with shaking until equilibrium is reached (in about 20 minutes).

At the end of this period the loose cups are shaken off the central vessels so that their contents mix with the sludge in the main part of the manometer flask, and both taps are closed.

The reading of both sides of the manometer is noted at once, and shaking is recommenced. Further readings are taken at hourly intervals, and the oxygen uptake of the material in the right-hand flask is calculated in the usual way.

If the results are graphed the slope of the curve gives a measure of the activity of the sample of sludge.

APPENDIX

REFERENCE TABLES AND GRAPHS FOR USE WITH THE VAN SLYKE MANOMETRI APPARATUS

(Reproduced by kind permission of Dr. Van Slyke and the *Journal of Biological Chemistry*)

TABLE I

FACTORS FOR CALCULATION OF CARBON DIOXIDE CONTENT OF BLOOD

	Factors by which Millimetres $P_{[CO_2]}$ are Multiplied to give:									
	Millimoles CO_2 per Litre of Blood					Vol. per cent. CO_2 in Blood				
Tempera-ture	Sample= 0.2 c.c.	Sample=1.0 c.c.				Sample= 0.2 c.c.	Sample=1.0 c.c.			
		S=3.5 c.c.		S=7.0 c.c.			S=3.5 c.c.		S=7.0 c.c.	
	S=2.0 c.c. a=0.5 c.c. i=1.037	a=0.5 c.c. i=1.037	a=2.0 c.c. i=1.017	a=0.5 c.c. i=1.037	a=2.0 c.c. i=1.017	S=2.0 c.c. a=0.5 c.c. i=1.037	a=0.5 c.c. i=1.037	a=2.0 c.c. i=1.017	a=0.5 c.c. i=1.037	a=2.0 c.c. i=1.017
°C.										
15	0.1514	0.0313	0.1229	0.0341	0.1335	0.3370	0.0697	0.2735	0.0758	0.2974
16	07	11	22	38	25	54	93	19	52	50
17	0.1499	10	15	35	15	38	89	04	46	28
18	92	08	08	33	06	22	86	0.2690	41	06
19	86	06	02	31	0.1297	07	82	75	36	0.2886
20	79	05	0.1196	28	88	0.3292	78	62	31	66
21	72	03	90	26	79	78	75	48	26	48
22	66	02	83	24	70	63	71	34	21	28
23	59	00	77	22	62	48	68	20	16	08
24	53	0.0299	71	19	53	34	65	07	11	0.2790
25	46	97	65	17	45	20	61	0.2594	07	72
26	40	96	60	15	37	06	58	81	02	53
27	34	94	54	13	29	0.3193	55	69	0.0698	36
28	28	93	49	11	22	79	52	57	93	20
29	22	91	43	10	15	66	49	45	89	04
30	16	90	38	08	08	53	46	33	85	0.2688
31	11	89	33	06	01	40	43	22	82	74
32	05	88	28	05	0.1195	28	40	11	78	59
33	00	86	23	03	88	15	37	00	74	44
34	0.1394	85	18	01	82	03	34	0.2489	71	30

To obtain factors for a sample other than 1 c.c., divide the above factors for 1 c.c. by the cubic centimetres of sample analysed: e.g. for a 2 c.c. sample, S, A, and *a* being the same, the factors are one-half of those for a 1 c.c. sample.

TABLE II

FACTORS FOR CALCULATION OF OXYGEN, CARBON MONOXIDE, OR NITROGEN CONTENT OF BLOOD

Factors by which Millimetres $P_{[O_2]}$, $P_{[CO]}$, or $P_{[N_2]}$ are Multiplied to give:

Millimoles O_2, CO or N_2 per Litre of Blood

Temperature °C.	Sample = 0.2 c.c. S=2.0 c.c. a=0.5 c.c. i=1.00	Sample = 0.5 c.c. S=2.0 c.c. a=0.5 c.c. i=1.00	Sample = 1 c.c. S=3.5 c.c. a=0.5 c.c. i=1.00	Sample = 1 c.c. S=3.5 c.c. a=2.0 c.c. i=1.00	Sample = 2 c.c. S=7 c.c. a=0.5 c.c. i=1.00	Sample = 2 c.c. S=7 c.c. a=2.0 c.c. i=1.00
15	0.1389	0.05556	0.02780	0.1113	0.01396	0.0558
16	84	38	70	09	90	56
17	80	20	61	05	85	54
18	75	00	51	01	80	52
19	70	0.05480	41	0.1097	75	50
20	65	60	31	93	70	48
21	60	40	21	89	65	46
22	55	20	11	85	60	44
23	50	00	02	81	55	42
24	45	0.05380	0.02692	77	50	40
25	40	60	83	74	45	38
26	35	40	73	70	41	36
27	31	22	64	67	36	34
28	26	04	55	63	31	32
29	22	0.05286	47	59	27	30
30	18	70	38	55	22	29
31	13	52	29	52	18	27
32	09	34	20	48	14	25
33	04	16	11	44	09	24
34	00	00	02	41	05	22

Volume per cent. O_2, CO or N_2 in Blood

Temperature °C.	Sample = 0.2 c.c. S=2.0 c.c. a=0.5 c.c. i=1.00	Sample = 0.5 c.c. S=2.0 c.c. a=0.5 c.c. i=1.00	Sample = 1 c.c. S=3.5 c.c. a=0.5 c.c. i=1.00	Sample = 1 c.c. S=3.5 c.c. a=2.0 c.c. i=1.00	Sample = 2 c.c. S=7 c.c. a=0.5 c.c. i=1.00	Sample = 2 c.c. S=7 c.c. a=2.0 c.c. i=1.00
15	0.312	0.1246	0.0623	0.2493	0.0317	0.1251
16	10	42	21	85	15	46
17	09	37	19	78	14	42
18	08	33	17	68	12	37
19	07	29	15	59	11	32
20	07	24	13	50	09	28
21	06	20	10	41	08	24
22	05	16	08	32	06	19
23	03	11	06	23	05	15
24	02	07	04	14	03	10
25	01	0.1203	02	06	02	06
26	00	0.1199	00	0.2398	01	02
27	0.299	95	0.0598	90	0.0299	0.1198
28	98	91	96	82	98	93
29	97	87	93	74	96	89
30	96	83	92	66	95	85
31	95	79	90	58	94	81
32	94	75	88	50	92	77
33	93	71	86	42	91	73
34	92	67	83	33	90	69

Here are given the corrections for dissolved gases to be subtracted when combined O_2 is to be estimated from total O_2.

TABLE III

FACTORS FOR ESTIMATION OF CARBON BY WET COMBUSTION

| Temperature | Factors | | | Vapour Tension of Water |
	Submicro-analysis $a=2\cdot000$ $S=3\cdot00$ $i=1\cdot016$	Micro-analysis $a=10\cdot00$ $S=3\cdot00$ $i=1\cdot007$	Macro-analysis $a=46\cdot00$ $S=4\cdot00$ $i=1\cdot000$	
° C.				mm.
10	0·001474	0·007303	0·03320	9·1
11	66	265	304	9·8
12	58	228	289	10·4
13	51	192	274	11·1
14	44	157	269	11·9
15	37	122	244	12·7
16	30	088	229	13·5
17	24	054	215	14·4
18	17	020	201	15·3
19	10	0·006987	187	16·3
20	03	954	173	17·4
21	0·001397	922	159	18·5
22	90	890	145	19·6
23	84	859	132	20·9
24	78	828	119	22·2
25	72	798	106	23·5
26	66	769	093	25·0
27	60	740	080	26·5
28	54	711	067	28·1
29	49	683	055	29·7
30	43	655	043	31·5
31	37	628	031	33·4
32	32	601	019	35·3
33	27	575	007	37·4
34	21	549	0·02996	39·5
35	16	523	985	41·8

When determining total plasma lipids by combustion, following Van Slyke, one may multiply the carbon factors by 1·266 in order to obtain factors for calculation of milligrams of total lipids directly from $P_{[CO_2]}$.

When cholesterol is determined by combustion of the digitonide, $C_{27}H_{46}O \cdot C_{55}H_{94}O_{28}$, one may multiply the above factors by 0·3926, in order to obtain a table of factors for calculating milligrams of cholesterol from $P_{[CO_2]}$.
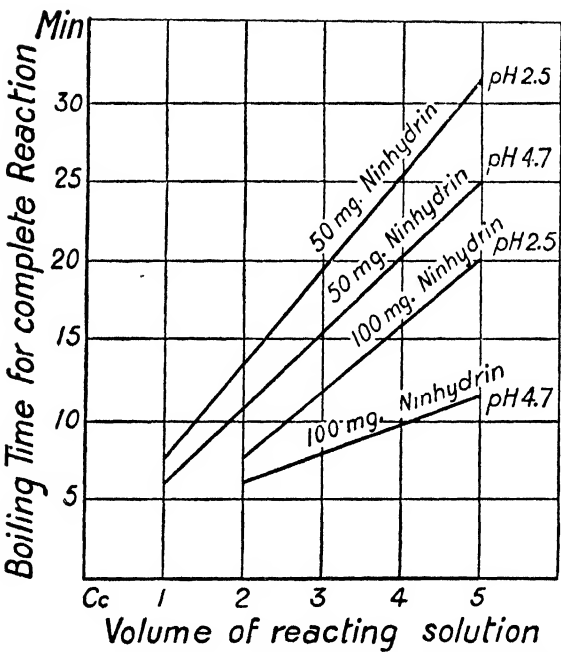
Fig. A.1.   Graph Giving Requisite Reaction Times for Determination of
Amino-acids by Use of Ninhydrin.

TABLE IV

FACTORS FOR CALCULATION OF "CARBOXYL CARBON" IN ESTIMATION OF AMINO-ACIDS BY USE OF NINHYDRIN

Tempera-ture	Carboxyl Carbon			Carboxyl Nitrogen*		
	†$a = 10$ $i = 1·007$	$a = 2$ $i = 1·017$	$a = 0·5$ $i = 1·037$	$a = 10$ $i = 1·007$	$a = 2$ $i = 1·017$	$a = 0·5$ $i = 1·037$
°C.						
15	0·007168	0·001447	0·0003688	0·008361	0·001688	0·0004303
16	133	39	69	320	79	0·0004280
17	097	32	50	278	71	58
18	061	25	32	237	62	37
19	026	18	14	196	54	16
20	0·006991	11	0·0003596	156	46	0·0004195
21	958	04	78	117	38	74
22	926	0·001397	61	078	30	54
23	893	91	44	040	23	35
24	860	84	28	003	15	16
25	827	78	12	0·007964	08	0·0004097
26	797	71	0·0003496	928	0·001599	79
27	767	65	80	893	92	60
28	736	59	65	858	85	42
29	706	53	50	822	78	25
30	676	47	35	787	71	07
31	648	42	20	755	66	0·0003990
32	621	36	06	723	59	74
33	593	30	0·0003392	690	52	57
34	566	25	78	658	46	41
35	538	20	64	626	40	25

* "Carboxyl nitrogen" = carboxyl carbon × 14·01/12·01.

† a is the volume in cubic centimetres at which $P_{[CO_2]}$ is measured, and i the correction factor for reabsorption of CO_2. The volume (S) of solution extracted in the Van Slyke-Neill chamber is 3 c.c. for all three types of analyses.

For the most precise results, the factors must, for each apparatus and type of analysis, be multiplied, either by the ratio:

$$\frac{\text{Actual } a \text{ found by calibration}}{a \text{ assumed in calculation of Table IV}}$$

or by the empirical b correction factor found in analyses of a pure amino-acid.
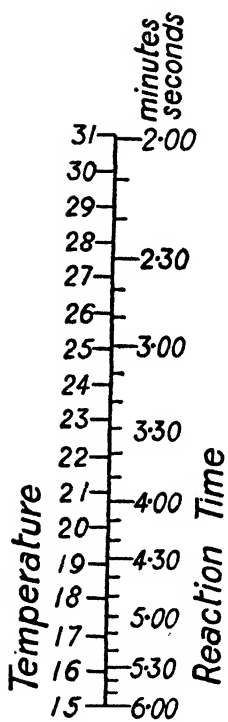
Fig. A.2.   Nomogram Giving Reaction Period for Complete Decomposition of
Amino-acids by Nitrous Acid.

TABLE V

FACTORS FOR CALCULATION OF AMINO NITROGEN

Temperature	Factors by which Millimetres of $P_{[N_2]}$ are Multiplied to give Milligrams of Amino N in Sample Analysed		Factors by which Millimetres of $P_{[N_2]}$ are Multiplied to give Milligrams of Amino N per 100 c.c. Blood when Filtrate Sample is Equivalent to 0·5 c.c. Blood
	$a = 0·5$ c.c.	$a = 2·0$ c.c.	$a = 0·5$ c.c.
° C.			
15	0·000390	0·001561	0·0780
16	389	55	777
17	387	49	774
18	386	44	772
19	385	38	769
20	383	33	766
21	382	27	763
22	380	22	761
23	379	16	758
24	378	11	756
25	376	06	753
26	375	00	750
27	374	0·001495	748
28	372	90	745
29	371	85	743
30	370	80	740
31	368	74	737
32	367	69	734
33	366	64	732
34	365	59	730

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